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PHYLOGENY OF LIPOPROTEIN RECEPTOR FUNCTION REVEALED BY STUDIES ON THE UPTAKE OF VITELLOGENIN INTO GROWING OOCYTES

by

STEFANO STIFANI

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

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ABSTRACT

In order to gain knowledge of the mechanisms that assure efficient vitellogenesis in birds, two distinct vitellogenin (VTG)-binding proteins were identified from chicken oocytes. The first of these receptors is a 95-kDa protein that recognizes its ligand by interacting with sites located within the aminoterminal region of VTG designated lipovitellin I. This receptor was shown to be functionally and immunologically very closely related to cognate VTG receptors of similar Mr's identified from frog and fish oocytes, indicating that regulation of vitellogenesis among nonmammalian vertebrates is accomplished through structurally conserved receptors required for internalization of VTG. The chicken oocyte 95-kDa VTG receptor was also shown to function as the receptor for apolipoprotein-B-containing lipoproteins, such as very low density lipoprotein, and to be immunologically related to the bovine LDL receptor, a property shared with the amphibian VTG receptor. Furthermore, direct binding of chicken and fish VTGs to the rat and bovine LDL receptors was demonstrated. The second chicken oocyte VTG-binding protein, having an Mr of ~350,000, exhibited properties that suggested it may represent in birds an oocytic version of the larger (~500 kDa) low density lipoprotein receptor-related protein (LRP), a molecule first identified in mammalian somatic tissues and which has been hypothesized to function as a receptor for apolipoprotein-E-containing lipoproteins, such as chylomicron remnants. Despite the absence of apolipoprotein-E in birds, a 500-kDa protein immunologically and functionally related to mammalian LRP was also identified for the first time in somatic tissues of these animals. Both of the chicken LRPs shared with their mammalian counterpart the ability to interact with VTG, suggesting that LRP is a multifunctional receptor. This notion was further supported by the demonstration that LRP is also a receptor for α_2-macroglobulin. The different tissue distribution of the chicken LRPs, particularly the specific expression of the smaller LRP in oocytes, and the demonstration that one is not a

proteolytic product of the other suggest that they perform similar but different tasks in oocytes and somatic cells, respectively. Altogether, these findings not only provided new and interesting insights into the process of vitellogenesis, but also established for the first time a direct evolutionary link between oocyte VTG receptors in oviparous species and members of the LDL receptor gene family in mammals.

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ABBREVIATIONS

Apo, apolipoprotein

BSA, bovine serum albumin

cAMP, cyclic adenosine 3':5' monophosphate

CURL, compartment of uncoupling of receptor and ligand

DAG, diacylglycerol

DEAE, dietylaminoethyl

dl, decilitre

EDTA, ethylenediaminotetraacetic acid

EGF, epidermal growth factor

EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FBS, fetal bovine serum

gp330, glycoprotein 330

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

kDa, kiloDaltons

IgG, immunoglobulin G

LDL, low density lipoprotein

LRP, low density lipoprotein receptor-related protein

LV, lipovitellin

α_2M, α_2-macroglobulin

α_2M:m, methylamine-activated α_2-macroglobulin

MEM, minimal essential medium

MPF, maturation promoting factor

M_r, molecular weight

MVB, multivesicular body

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate-buffered saline

PMSF, phenylmethylsulfonyl fluoride

PV, phosvitin

R/O, restricted ovulator

SDS, sodium dodecyl sulfate

VLDL, very low density lipoprotein

β-VLDL, β-migrating very low density lipoprotein

VTG, vitellogenin

Chapter I

INTRODUCTION

Embryonic development among oviparous (egg-laying) animals takes place within the confinement of a laid egg. This implies that the growing embryo relies on the components stored within the egg during oogenesis as its only source of nutrients . Thus, the generation of viable off-spring is dependent on the accumulation of the necessary nourishment prior to fertilization. Yolk, the complex storage component of the egg, serves as the major food source. Formation of the yolk reserve during the growth of oocytes into mature eggs is accomplished by deposition of massive amounts of plasma-derived molecules into the growing oocytes, a process named vitellogenesis. Most, if not all, yolk proteins are synthesized outside the ovary, predominantly in the liver in vertebrates and in various organs in invertebrates; synthesis is under the regulation of hormones such as estrogen or ecdysone. After secretion into the circulatory system, these yolk precursors are transported to the ovary where they are recognized by specific oocyte plasma membrane receptors and internalized by receptor-mediated endocytosis. Without selective and efficient transport of these molecules into the oocytes, the dramatic growth in size undergone by these cells in preparation for fertilization could not take place and the nutritive requirements for embryonic development could not be met.

Given the fundamental role played by the oocyte plasma membrane receptors involved in the internalization of yolk precursors, it was the scope of the work presented in this thesis to perform experiments aimed at gaining a detailed knowledge of the properties of one particular type of yolk precursor receptor, namely the vitellogenin receptor. Vitellogenin is by far the most important yolk precursor protein in invertebrate and lower vertebrate oviparous animals, where it provides the majority of the protein mass of the fully grown egg. In avian species, on the other hand, vitellogenin shares almost equally the task of building the bulk of the yolk with very low density lipoprotein.

This thesis describes the results obtained from studies mainly directed at investigating the features of receptors involved in vitellogenin uptake by the oocytes of the chicken, *Gallus domesticus*. The choice of the chicken as premiere animal system was dictated by the observation that i) very large amounts of vitellogenin, as well as very low density lipoprotein, are present in the blood of mature females or estrogen-treated males, thus enormously facilitating the isolation of these proteins, and ii) contrary to other oviparous animals, chickens lay eggs regularly throughout the year and their oocytes undergo an enormous growth rate in the final days before oviposition (corresponding to an active engagement in receptor-mediated endocytosis), thus making way for the biochemical characterization of the molecules of interest.

In order to provide the reader with information necessary to evaluate the experimental work presented in this thesis, the following pages are intended to offer a summary of the prominent aspects of egg development in birds. Because knowledge of the many stages of this complex process has been pieced together from studies performed using different animal models, key experiments and observations pertinent to species other than birds will also be described. In addition, a summary of the salient features of the process of receptor-mediated endocytosis will also be provided.

I. GENERAL ASPECTS OF OOCYTE GROWTH

I.A. Oogenesis

The production of viable eggs, an essential feature of the reproductive effort of all oviparous animals, is the result of a fascinating sequence of events providing one of the most striking examples of regulation of cell growth. With minor differences, the various sequential processes involved in regulation of egg development (**oogenesis**) are very similar in different species (1,2). Oocytes are derived from **oogonia**, a mitotically dividing cell population that develops from primordial germ cells that migrate into the ovary early in embryogenesis. After undergoing mitotic proliferation for some time within the ovary, oogonia enter meiosis. The initial events of meiosis (pre-leptotene, leptotene, and zygotene) take place normally so that DNA replicates and homologous chromosomes pair and begin to condense. However, after these steps the cells remain arrested in prophase of division I of meiosis and the chromosomes take on a "lampbrush" configuration (diplotene), corresponding to busy engagement in RNA synthesis. The arrested cells are referred to as **primary oocytes**. Primary oocytes then begin a period of growth which varies in length depending upon different species. It is during this time that primary oocytes accumulate all the components of the complex machinery that will later on direct early embryonic development, such as ribosomes, tRNAs, cytoplasmic organelles, mRNAs, as well as the yolk, the protein- and lipid-rich nutritional reserve that will provide the necessary source of nutrients for the embryo within the enclosed environment of the egg. In birds this growth period is characterized by an initial slow phase lasting months to years during which no yolk is deposited. This is then followed by rapid yolk formation over a period of approximately two months, with a very rapid growth phase during the last week before ovulation when the majority of the yolk is deposited (3).

The dramatic growth undergone by primary oocytes is the result of the combined storage within these cells of both endogenously and exogenously synthesized products. In the first place, primary oocytes take advantage of the presence of duplicate sets of diploid chromosomes resulting from arrested meiosis. This provides them with double the amount of DNA available for RNA synthesis. In the second place, some of the proteins synthesized by the oocyte contribute enormously to cell growth by mediating incorporation of macromolecules synthesized outside the oocyte. It is the formidable avidity of growing oocytes for certain circulating macromolecules, particularly lipoproteins, that is responsible for the fast and large growth of these cells during the final stages of oogenesis. The process of rapid uptake of precursors of yolk components is termed **vitellogenesis.**

I.B. Vitellogenesis

During vitellogenesis, oocytes grow mainly as the result of the accumulation of large reserves of proteins and lipids. Chicken oocytes, for example, can incorporate 1.0 - 1.5 grams of protein per day during the last seven days before oviposition (3,4). Synthesis of the major yolk precursors is under the control of estrogenic hormones in nonmammalian vertebrates (5) and of the juvenile hormone and ecdysone in insects (6). In the former, estrogenic hormones produced by the ovary in response to gonadotropins are secreted into the bloodstream and carried to the liver where they regulate the synthesis of several macromolecules that serve as precursors of yolk components. Experimental evidence showed that in almost all oviparous animals, from birds to nematodes, vitellogenin (VTG), a lipophosphoglycoprotein with calcium-binding properties, is the major yolk precursor (7-9). In laying hens, for example, estrogen induces circulating VTG concentrations of up to 2000 - 2500 mg/decilitre (dl) (2). In vertebrate species, vitellogenins are dimeric proteins with individual subunits having molecular weights ranging from 180,000 to 240,000 (10,11). They have been conserved during evolution: when sequence information became available, as is the case for vitellogenins from chickens (12), the amphibian, *Xenopus laevis* (13), and the nematode, *Caenorhabditis elegans* (14), it became evident that VTGs from different species are closely related. A structural similarity is also apparent when considering the events following internalization by the oocytes. All vitellogenins studied to date in nonmammalian vertebrates are proteolytically cleaved, after uptake, into two distinct groups of yolk proteins, termed lipovitellin (LV) and phosvitin (PV) (10,11,15).

These cleavage products of vitellogenin remain stored until embryogenesis starts, after which they are utilized as a source of amino acids, lipids, sugar, inorganic phosphate, and calcium. Fig. I 1 provides a schematic representation of the process of synthesis, uptake into oocytes, and partial proteolysis of VTG in nonmammalian vertebrates.

In amphibia, vitellogenin is the major yolk-related protein and provides 80-85% of the total yolk proteins (2). In birds, triglyceride-rich lipoproteins of the very low density class (VLDL) are the major yolk precursors together with vitellogenin (16,17). Additional components isolated from the yolk of vertebrate eggs, such as transferrin (18), riboflavin-binding protein (19), retinol-binding protein (20), and immunoglobulins (21) share structural and immunological properties with corresponding plasma proteins and are presumably also taken up into the oocytes by processes involving specific receptors. In some invertebrate species, such as the insect, *Drosophila melanogaster*, yolk proteins (*i.e.* vitellogenin) can also be synthesized within the ovary itself, by cells other than the oocytes. Such cells, normally referred to as **follicle cells**, are part, together with the oocyte, of complex structures known as **follicles**. Follicles constitute individual developing entities within the ovary and their development follows the growth of oocytes until the very last moments prior to ovulation.

I.C. Folliculogenesis

During oogenesis, in different ways and at different stages depending on which species is considered (22,23), oocytes become invested with a particular type of accessory cells named follicle cells. These cells help nourish the developing oocytes and are actively involved in regulating oocyte maturation, the last stage of oocyte development. In the case of *Drosophila melanogaster*, a particular type of follicle cells named nurse cells derive from the same oogonium that gives rise to an oocyte. This is possible because, after the oogonium undergoes four mitotic divisions to generate 16 cells, only one of these eventually becomes an oocyte, while the remaining cells become nurse cells (24). Studies performed with the pipefish, *Syngnathus scovelli*, have similarly suggested that both primary oocytes and prefollicle cells derive from a single precursor, the luminal epithelium (23). Follicle cells, however, are not the only structure surrounding growing oocytes. In vertebrate animals, by the end of the relatively slow primary growth stage preceding vitellogenesis, an oocyte is part of a complex multilayered structure termed a follicle. The chicken ovarian follicle is characterized by an oocyte surrounded by a sheath of follicle cells (specifically termed **granulosa cells**) which are separated from the oocyte surface by an acellular layer termed the **perivitelline layer**. The perivitelline layer is crossed by long microvillar processes extending from the oocyte surface to the granulosa cells. The granulosa cells, in turn, are embedded between the perivitelline layer and a collagenous **basement membrane** (also called basal lamina). Next to the basement membrane is a connective tissue layer which is highly vascularized and is designated the **theca**. The theca is composed primarily of fibroblast-like cells interspersed within the connective tissue fibers aggregated into loosely packed bundles (3).

Follicle cells are extremely important during egg development. Granulosa cells are actively involved in secretion of progesterone, a hormone that plays a major role in oocyte maturation (3). Theca cells, on the other hand, secrete estrogen which, as previously described, stimulates hepatic synthesis of the yolk precursors (3,5). In addition, follicle cells help the growing oocytes by supplying small precursor molecules that can be used for synthesizing larger molecules. This is made possible by the existence of gap junctions between the oocyte surface and the follicle cell microvilli (25,26), through which exchange of small molecules is accomplished.

Arguably the most important role of follicle cells is their ability to release oocytes from their arrested state at prophase of meiotic division I. The ensuing resumption of meiosis triggers the final stage of egg formation, named **oocyte maturation.**

I.D. Oocyte maturation

Before an oocyte can become a fertilizable egg, it must move past meiosis I before arresting again, as a **secondary oocyte**, at metaphase of division II of meiosis. It will then remain arrested until fertilization triggers the completion of meiosis. Oocyte maturation has been studied most extensively in amphibia, where it has been shown that resumption of meiosis is initiated when gonadotropins induce ovarian follicle cells to release progesterone (1). Following its release, progesterone is believed to interact with oocyte surface receptors (27), and to trigger intracellular responses that, according to molecular details that remain to be fully elucidated, result in decreased levels of the two very important second messengers, cyclicAMP (cAMP) and diacylglycerol (DAG) (28,29). Based on the observation that both cAMP and DAG regulate protein kinases (protein kinase A and C, respectively) it was first suggested (30) and then demonstrated (31) that phosphoproteins are involved in maintaining meiotic arrest. In particular, the discovery that mature frog oocytes contain high levels of the so-called maturation-promoting factor (MPF) relative to primary oocytes (31) and that MPF isolated from the latter is phosphorylated (32,33) suggested that MPF is required for the exit from meiotic prophase I. MPF isolated from frog oocytes has been shown to be composed of two separate polypeptide chains (34). One component, of molecular weight 34,000, has been identified as the frog homolog of a fission yeast protein encoded by the gene cdc2 which is required for regulation of the cell-cycle. The cdc2 protein ($p34^{cdc2}$) is a serine/threonine protein kinase that can phosphorylate a number of substrates but exhibits strong preference for histone H1. The second component of MPF, a protein of 45,000 Daltons belonging to the family of proteins named cyclins, can also be phosphorylated by $p34^{cdc2}$ (35). Cyclins were first identified in sea urchin embryos, where they were shown to accumulate during interphase and to disappear near the end of each mitosis (36), suggesting a role in regulation of the cell cycle. The role of cyclins in oocyte maturation was shown when injection of cyclin mRNA into arrested *Xenopus* oocytes resulted in resumption of meiosis (37). Based on the available evidence, a model has been proposed for MPF activation in which the first step is the interaction of progesterone with a putative oocyte plasma membrane receptor that has been tentatively identified as a 110-kDa protein (27). This results in inactivation of serine/threonine protein kinases that normally maintain $p34^{cdc2}$ in a highly phosphorylated state. Concurrently, expression of cyclin is turned on and $p34^{cdc2}$, still phosphorylated on tyrosine residues, is able to interact with and phosphorylate cyclin. The interaction with cyclin, in turn, prevents continued phosphorylation of $p34^{cdc2}$ by a tyrosine kinase and the complex of cyclin and fully dephosphorylated $p34^{cdc2}$ constitutes active MPF (29). The process is autocatalytic since MPF can activate more molecules of MPF, thus potentiating the response. Activation of MPF leads to activation of protein kinases that can phosphorylate nuclear lamins and chromosomal proteins, causing the nuclear envelope to break down and chromosomes to condense, thereby driving the cell to metaphase. The amphibian oocyte is now ready to ovulate from its follicular investment and become a fertilizable egg. Oocyte maturation in other vertebrate and invertebrate species also seems to proceed according to the aforementioned model (29).

II. THE ROLE OF VITELLOGENIN IN OOCYTE GROWTH

II.A. Precursor-product relationship between vitellogenin and the yolk proteins, lipovitellin and phosvitin.

Although demonstration of the hepatic origin of the major proteins of the egg yolk was provided early after their characterization (for review see Ref. 2), demonstration of the relationship between the yolk proteins, lipovitellin and phosvitin, and a single plasmatic precursor, vitellogenin, only came several years later at the end of a series of investigations starting from studies of the composition of yolk proteins from hen's eggs. Egg yolk proteins were separated, on the basis of their density, into a high density and a low density fraction (38,39). The high density fraction was shown to contain a limited number of proteins, ~65% of which consisted of phosvitin (~15%) (40) and lipovitellin (~50%) (41-43). Subsequent findings indicated that neither phosvitin nor lipovitellin are homogeneous proteins. Bernardi and Cook showed that lipovitellin could be resolved into two components, termed α- and ß-lipovitellin (44). They are lipophosphoproteins, containing 20% (by weight) lipid. In their native state lipovitellins are dimers of ~400 kDa that dissociate into monomeric subunits under alkaline conditions. The two avian LVs differ in their subunit composition; whereas both α- and ß-lipovitellin contain heavy chains (termed LV I) of greater than 100,000 Daltons and light chains (termed LV II) of molecular weight smaller than 40,000 (45), α-LV has been shown to contain additional components of intermediate size (46). The native (dimer) α- and ß-lipovitellins have the same lipid content, primarily phospholipid (47), the same nitrogen content, and molecular weight, but different phosphorus content, chromatographic behaviour and dissociative characteristics (48,49).

In a similar way, phosvitin was fractionated into at least two components (50,51). Both components are glycophosphoproteins of highly unusual amino acid composition; approximately 50% of their amino acid residues are serine, almost all of which is phosphorylated. Both phosvitins contain ~10% protein-phosphorus, but differ in size (40 kDa and 36 kDa, respectively) and amino acid composition (52-54).

Because of the presence of lipovitellin and phosvitin in egg yolk in separable forms, the initial model was that they represented the products of two different genes expressed in the liver (55), and thus they could be found in the blood in the same form as in the egg yolk (56). This notion was proven to be wrong when attempts to isolate free phosvitin from the blood of laying hens resulted in the isolation of a complex of phosvitin and lipovitellin (57). A similar complex was also isolated from the blood of mature females and estrogen-treated males of the amphibian, *Xenopus laevis*. Such complexes could be dissociated under appropriate conditions into the yolk proteins, lipovitellin and phosvitin (58). Following a nomenclature introduced in 1969 to indicate female-specific yolk precursors in insects (59), the name vitellogenin was adopted for the avian and amphibian proteins (60). However, the precursor-product relationship between vitellogenin and lipovitellin and phosvitin could only be demonstrated convincingly when it was shown by gel electrophoresis that both the *Xenopus* and the chicken vitellogenin complexes were composed of a single polypeptide chain when appropriate measures were taken in order to prevent proteolysis during the isolation procedure (10,11,15). This led to the demonstration of the dimeric nature of vitellogenins and their role as parent molecules of lipovitellins and phosvitins.

As the study of vitellogenins progressed, new findings appeared to indicate that the picture was far more complex than initially thought. Work by Wahli and associates showed that different cDNA sequences could be isolated from *Xenopus laevis* liver corresponding to four distinct mRNA species coding for VTG (61,62). The demonstration that each individual cDNA sequence hybridized to different restriction fragments of genomic DNA, together with heteroduplex mapping in the electron microscope and heteroduplex melting experiments, indicated that vitellogenin in *Xenopus* is encoded by a small family of genes (63). Those four genes have been shown to have close sequence relatedness, suggesting that they arose from a common ancestor (64,65). As an apparent consequence of the presence of a family of vitellogenin genes, at least three distinct forms of VTG can be detected in the blood of *Xenopus laevis* animals (66). In turn, this heterogeneity of the VTG population results in the heterogeneity of the yolk proteins: three different forms of both the high and the low molecular weight subunits of lipovitellin, two different phosvitins and two smaller, phosvitin-like peptides, termed phosvettes, were identified (67).

In the chicken, only one VTG gene has so far been described in its entirety (68,69). Nevertheless, three different vitellogenin polypeptides have been isolated from the blood of mature females; they have been designated VTG I, II, and III (46,70). Several lines of evidence suggested that the three variants of avian vitellogenin are products of different genes, including different amino acid compositions, phosphorus contents, and peptide mapping patterns. The ratio of VTG I, II, and III in the circulation was found to be 0.33:1.0:0.08, respectively (70). In analogy to the situation observed in *Xenopus* oocytes, the population of avian yolk proteins derived from vitellogenin is highly heterogeneous (71). At least five different polypeptide chains can be identified after isolation of the lipovitellin fraction and separation of its components by electrophoresis on a SDS-polyacrylamide gel (72). Similarly, there are at least five phosphoproteins in chicken egg yolk, two phosvitins and three phosvettes, termed phosvette E1, E2 , and F (73).

The heterogeneity of the *Xenopus* and chicken VTG families is not a characteristic feature of nonmammalian vertebrate species alone and can also be observed in invertebrates. When a larger number of oviparous species was investigated, it became apparent that the presence of several VTG genes is a common feature among these animals.

II.B. The vitellogenin gene family

Although the site of delivery of VTG, namely the growing oocytes, is invariant in all species, its site of synthesis varies depending on different animals. In insects, for example, VTG is produced in the female fat body under the regulation of juvenile hormone and ecdysone (74). In nematodes, synthesis takes place in the intestine of the hermaphrodites (75), and in the sea urchin VTG appears to be synthesized in the intestine and gonads of adults of both sexes (76) (although the possibility remains that synthesis in male animals may have been induced by traces of estrogenic hormones present in their food supplements). Regardless of where synthesis occurs, a common theme has become apparent: VTGs are encoded by small gene families. We have already seen that four VTG genes exist in *Xenopus laevis* (63). Of these, only the gene designated A2 has been fully sequenced and shown to encode LVI, LVII, and either one phosvitin or two phosvettes (13). In chickens, only the gene encoding the most abundant product, VTG II, has been sequenced in its entirety: it encodes LVI, LVII and the larger of the two phosvitins (12). Partial sequence information has recently become available for the region of the gene encoding VTG III that corresponds to a phosvette polypeptide (77). In *C. elegans*, the VTG gene family comprises six genes (78) and one of them, designated *vit-5*, has been sequenced (14). Two classes of vitellogenins can be found in nematodes: one consists of 170-kDa proteins that are not cleaved into smaller yolk proteins and the second one consists of 115- and 88-kDa VTGs that are derived from a short-lived 180-kDa precursor (14). In *Drosophila*, three VTG-encoding genes have been identified and sequenced: they produce smaller proteins of 45-kDa that are designated yolk protein 1,2, and 3 (79).

Comparison of the complete nucleotide and amino acid sequences of chicken, *Xenopus*, and *C. elegans* VTGs revealed that these proteins are related (80). In particular, the chicken and the amphibian VTG genes share the same exon-intron organization and show extensive homology; the nematode gene, on the other hand, has a smaller number of introns and exhibits lower sequence conservation. It is significant that the regions most conserved between *Xenopus* and chicken vitellogenins are also those that are most conserved between *Xenopus* and *C. elegans*. On the other hand, a striking difference between the two vertebrate and the nematode VTGs is the absence of the phosvitin region in the latter. In line with this observation, this domain is the least conserved between chicken and frog VTGs, suggesting that no considerable selective pressure has been exerted upon it during evolution. Assuming that those regions of VTG involved in regulation of important events during vitellogenesis have been conserved along phylogenetic lines, it can be suggested that the phosvitin domain is likely not directly involved in events preceding internalization into growing oocytes. It is tempting to speculate that the presence of the phosphate- and calcium-carrying phosvitin domain in vertebrate VTGs is compatible with a role for this domain in the process of bone formation (87). In this context, the phosphorus content of chicken VTG is higher than that of *Xenopus* VTG.

In summary, vitellogenins from species that diverged hundreds of millions years ago have maintained several common features. This is indicative of the possible participation of such conserved regions in interactions aimed at i) stabilizing the biologically active conformation of the molecules, ii) allowing specific interactions with oocyte surface receptors, and iii) directing correct intraoocytic processing and storage. The salient characteristics of vertebrate vitellogenins are summarized in Table I 1.

III. CONTRIBUTION TO OOCYTE GROWTH BY OTHER EGG-RELATED PROTEINS

The constituents of chicken egg yolk can be crudely separated by partially diluting the yolk and recovering, after a brief centrifugation, a particulate fraction and a supernatant fraction. As previously mentioned, the particulate fraction contains lipovitellin and phosvitin. The supernatant fraction can be further separated by centrifugation into a low density fraction and a subnatant of soluble proteins, termed livetins (42).

The low density fraction contains ~85% lipids (primarily neutral lipids) and is the major component of chicken egg yolk, making up approximately 70% of the yolk solids (47). When the polypeptide chains associated with the yolk low density fraction were characterized (81), a precise precursor-product relationship was demonstrated between plasma VLDL apoproteins and the yolk low density fraction, based on comparison of amino acid composition and immunological studies (82). Chicken VLDL isolated from the plasma of laying females contains two major apoproteins, known as apolipoprotein-B (apo-B) and apolipoprotein-VLDL-II (apo-VLDL-II) (82). Apo-VLDL-II is a disulfide-linked homodimer of identical 82-residue subunits. The molar ratio of apo-B to apo-VLDL-II in the plasma of estrogen-treated roosters was found to be 1:32, suggesting that these two components represent ~62% and ~38%, respectively, of the total VLDL protein mass, assuming a molecular weight of 500,000 for apo-B and 9,500 for apo-VLDL-II (83). Although the chicken apo-B gene has not been entirely sequenced, the size of its product has been estimated by comparison to the homologous human apo-B gene product (84). Contrary to VTG, which is only found in mature females, plasma VLDL can be detected in roosters or immature hens (85). However, rather low levels are present, reported ranging from less than 30 mg/dl to 60 mg/dl (81,85). These values are certainly very low when compared with VLDL levels in laying hens or estrogen-treated males which can be as high as 2000 mg/dl (86). In addition, VLDL from roosters and immature hens does not contain any trace of apo-VLDL-II, whose synthesis is turned on by estrogen according to mechanisms also involved in regulating expression of the VTG genes (reviewed in ref. 87). It is believed that in the absence of growing oocytes (a situation paralleled by the absence of apo-VLDL-II synthesis) there is no pressure on the animal to accumulate triglyceride-rich particles and lipoprotein lipase activities localized in various extrahepatic tissues effectively convert VLDL particles into LDL particles. When the rapidly growing oocytes require massive deposition of yolk precursors, on the other hand, the nutrient-rich VLDL particles are stabilized and prevented from lipolysis by the presence of apo-VLDL-II. Such hypothesis has been confirmed by recent studies that have shown that apo-VLDL-II can function as an inhibitor of lipoprotein lipase (88). Unlike apo-VLDL-

II, which is only synthesized in the liver, apo-B is also synthesized by kidneys and intestine (89). However, while apo-B synthesis in the liver exhibits both estrogen-dependent and estrogen-independent modes of expression, levels of synthesis are not modulated by estrogen in the other tissues (2).

The livetins, approximately 10% of the total yolk solids, are a heterogeneous population of proteins related to plasma proteins such as albumin, transferrin, vitamin-binding proteins, α_2-glycoproteins, and others (2). Their presence inside the oocyte results in some cases from selective transport mechanisms (18-21) but in other cases it is the result of nonspecific uptake processes, mainly pinocytosis (2).

The presence of different plasma-derived components within the yolk raises the interesting question of whether the relative composition of the yolk proteins during vitellogenesis is a reflection of the relative composition of the yolk precursors in the blood or varies at different stages independently of the situation in the blood. Although this interesting aspect has not been extensively investigated, some studies (90) indicated that the proportion of low density fraction increases rapidly initially and then declines as yolks near maturity, whereas both the livetins and lipovitellin/phosvitin complexes accumulate following the opposite trend.

IV. UPTAKE OF VITELLOGENIN BY THE OVARY

IV.A. The mechanism of receptor-mediated endocytosis

The first indication that a receptor-mediated process is involved in removal of VTG from the circulation came from studies showing that, in the presence of a functioning ovary, VTG is removed from the blood much more readily than in the absence of an ovary (as in the case of estrogen-treated male animals) (91,92). When compared with other plasma proteins, such as albumin, the rate of uptake of vitellogenin by growing oocytes is 25-50 times more rapid, on a molar basis, and displays the characteristics of a receptor-mediated process, being specific and saturable (93). Subsequent studies provided evidence that VTG, as well as other yolk precursors, can reach the oocyte surface by penetrating through channels separating adjacent granulosa cells and crossing the perivitelline layer (17,94). Once bloodborne proteins reach the oocyte plasma membrane, they are taken up by receptor-mediated endocytosis.

The process of receptor-mediated endocytosis has been extensively studied and characterized during investigations aimed at delineating the mechanisms involved in the sequential cell surface binding, internalization, and intracellular degradation of low density lipoprotein (LDL). This process (extensively reviewed in ref. 95) is initiated when specific receptors on the cell surface bind to LDL particles carried by the blood. This interaction is quickly followed by internalization of the ligand/receptor complexes, accomplished through the invagination and subsequent pinching-off of the region of the plasma membrane where the complexes are localized. This key step can only take place when the receptor molecules that bind LDL are localized at specialized regions where the cell membrane is indented to form craters. Not only the membrane is invaginated, but its inner surface is also coated with dense material resulting from the presence of several tightly interacting copies of the protein clathrin, hence the name "coated pits" given to these indented regions. Because coated pits have the ability to pouch inward into the cell and pinch off from the surface to form membrane-enclosed sacs named coated vesicles, LDL receptors that are localized at such pits are also transported inside the cell, carrying with them their load of bound LDL. After internalization, coated vesicles (which are now completely surrounded by their clathrin coat) progress through a series of stages including loss of the clathrin coats, fusion with other uncoated vesicles to form larger structures termed endosomes, and acidification of the endosomal compartment. Within endosomes the LDL and its receptor part company and while the receptor is recycled to the cell surface, LDL is delivered to a lysosome, where it is degraded. After returning to the cell surface, the receptor can once again home in coated pits, bind another LDL particle and initiate another cycle of endocytosis.

During the last decade, receptor-mediated endocytosis has been recognized as a mechanism by which cells internalize many macromolecules in addition to LDL and many of the most relevant aspects of the process have been studied in detail. For example, an interesting question is whether binding of ligand is the signal that induces receptors to associate with coated pits or such association takes place spontaneously even in the absence of ligand. There are examples of both cases. Whereas LDL receptors (96), as well as other receptors such as those for transferrin (97) or insulin (98), have been shown to move to coated pits in the absence of ligands, other receptors, such as the epidermal growth factor (EGF) receptor (99), are not trapped in coated pits unless occupied with ligands. The mechanisms by which receptors cluster in coated pits are still largely unknown. Studies of the LDL receptor have provided some insight into this interesting aspect. Certain mutant genes produce LDL receptors that reach the cell surface and bind the lipoprotein normally, but fail to cluster in coated pits and thus cannot be internalized together with the bound LDL (100). Mutations responsible for this phenotype were mapped at a region of the gene encoding the cytoplasmic domain of the receptor. A specific role for the cytoplasmic domain of the molecule in mediating internalization of the LDL receptor has been confirmed by *in vitro* studies where mutations were introduced through oligonucleotide-directed mutagenesis (101,102). Such analyses have indicated that the sequence, **NPXY** (with **X** representing any amino acid) is crucial for internalization of the receptor. However, an understanding of the cellular machinery involved in protein internalization still remains far from being pieced together.

After internalization, dissociation of ligands from their receptors takes place in endosomes, pleomorphic structures exhibiting a buoyant density that is much lower than that of lysosomes (103). The fluid within endosomes is slightly acidic (pH 5.0-6.0), probably due to the action of an ATP-dependent proton pump found associated with coated vesicles (104). Because LDL, as well as other ligands, has been shown to dissociate from its receptor if the pH is below 6.0, the acidic fluid of endosomes is expected to cause ligand-receptor dissociation. Following dissociation, the ligand moves to lysosomes while the receptor returns to the cell surface. Geuze and coworkers (105) made some observations that help to explain how ligands and receptors are directed to different destinations after dissociating in endosomes during their studies of the asialoglycoprotein receptor. A specific population of endocytic vesicles, designated CURL (Compartment of Uncoupling of Receptor and Ligand), exhibits long tubular extensions. Within these structures, internalized receptors were found attached to the membrane of the tubular extensions, while the ligands were found free in the lumen of the vesicle. These observations suggest that receptors return to the cell surface because they segregate in the tubular extensions and can be incorporated into small vesicles budding from the tubular membrane from which the luminal content is excluded. Because the ends of the tubular extensions are frequently coated with clathrin, it is possible that receptors are segregated away from the luminal content of endosomes by their interaction with the coated regions of the CURLs.

The end result of receptor-mediated internalization of LDL is the delivery of the lipoprotein to lysosomes, where hydrolytic enzymes degrade its polypeptide chains and allow delivery of the cholesterol load by removing fatty acyl chains from cholesterylesters. The delivered cholesterol is then utilized by the cell and participates in regulating correct intracellular cholesterol levels (95). In other cases, however, the situation is different. For example, after internalization and delivery to endosomes, transferrin dissociates from its receptor but does not progress to lysosomes. Rather, it recycles back to the extracellular environment after delivering its iron load (95).

IV.B. Uptake of vitellogenin by receptor-mediated endocytosis

Several *in vivo* studies provided evidence that the uptake of VTG by growing oocytes has the features of a receptor-mediated process, namely saturability and selectivity (92). Furthermore, elegant morphologic studies demonstrated that virtually the entire oocyte surface is populated with coated pits (106,107). When experimental protocols were devised for growing *Xenopus* oocytes *in vitro*, investigations on the interaction of VTG with intact cells provided some biochemical information regarding the nature of the interaction of vitellogenin with its receptor (93,108), including sensitivity to pH, ionic composition of the medium, and requirement for calcium ions. Such studies, however, failed to provide a direct molecular characterization of the VTG receptor. Attempts in that direction were made by Roth and coworkers, who investigated the interaction of chicken VTG with detergent extracts of membranes from chicken oocytes (109-111). While these authors demonstrated saturable and specific binding of VTG to solubilized oocyte membranes, special care should be taken when critically evaluating their results on the basis of the following observation. As mentioned earlier, the dissociation of the internalized VTG from its receptor is believed to occur within the lumen of endosomes, where the slightly acidic pH (pH 5.0-6.0) is expected to promote separation of the two molecules. The studies by Roth *et al.* with solubilized chicken oocyte membranes were performed at pH 6.0, a value that should not favor ligand-receptor interactions. The choice of this experimental pH was also not in keeping with results obtained from studies on VTG binding to *Xenopus* oocytes (108), which had indicated optimal binding conditions in the pH range 7.5-8.0. Thus, prior to the start of this thesis, very little was known about the molecular properties of the VTG receptors. It must be emphasized, however, that during the time necessary for the completion of this thesis, some reports have appeared in the literature describing studies concerning characterization of VTG receptors in various species. Reference to such studies will be given in the chapters describing the results of this thesis.

IV.C. Events occurring after VTG internalization

After uptake and cleavage of vitellogenin into the smaller yolk proteins, lipovitellin and phosvitin, these products are stored in a crystalline form within membrane-bounded yolk **platelets**, or **granules** (10). In *Xenopus laevis* oocytes, platelets occupy approximately 50% of the total volume and contain over 80% of the total protein mass of a fully grown oocyte (2). The proteolytic processing of VTG occurs quite rapidly after endocytosis, but no further proteolysis takes place so that lipovitellin and phosvitin remain stable during the entire period of oocyte growth (112). In contrast to the normal fate of endocytosed VTG, when the protein is injected directly into oocytes nonspecific and complete degradation takes place within 6-14 hr (113). These observations suggest that correct processing of VTG does not take place in the cytoplasm but is carried out within closed compartments not accessible to injected tracers. Work by Wallace and coworkers has thrown some light on the post-endocytic fate of VTG in *Xenopus* oocytes. Vitellogenin receptors are internalized regardless of occupancy (114), thus resembling the behavior of LDL receptors. After internalization, coated vesicles lose their clathrin coat and fuse to form endosomes. Endosomes containing vitellogenin tend to aggregate with one another forming so-called multivesicular bodies (MVBs). During the time it spends within MVBs, VTG dissociates from its receptor and is cleaved into the yolk proteins. The consequent liberation of the receptor results in the fusion of MVBs to form platelets of light density (115). Light yolk platelets are then converted into high density platelets in a process that has been shown to be dependent on the extracellular concentration of VTG. The majority of light platelets are not converted into heavy platelets if vitellogenin is not available or present at subsaturating concentrations (115). The proteolytic conversion of vitellogenin into lipovitellin and phosvitin has recently been shown to be performed by a pepstatin A-inhibitable enzymatic activity, very likely corresponding to the aspartyl protease, cathepsin D (116).

The post-endocytic pathway of VTG is different from that described for the uptake of LDL, where the internalized material is degraded in lysosomes; typical lysosomes have not been detected in vitellogenic oocytes (2) and this is probably the reason for the limited proteolysis of vitellogenin, as well as of other internalized proteins.

V. THE LDL RECEPTOR GENE FAMILY

V.A. The LDL receptor

Extensive studies of the LDL receptor locus (reviewed in ref. 117) have provided ample evidence that the structural organization of this protein is a reflection of the exon-intron organization of its gene. The LDL receptor is a multi-domain protein that results from the sequential arrangement of individual modules that, either alone or in combination with other modules, are encoded by single exons. The mature protein can be described in terms of five major domains which, from the amino (NH_2)- to the carboxyl (COOH)-terminus, are designated: 1) the ligand binding domain, 2) the EGF precursor-like domain, 3) the O-linked sugar domain, 4) the trans-membrane domain, and 5) the intracellular domain. Fig. I 2 provides a schematic model of the LDL receptor molecule.

V.A.1. The ligand binding domain

The modular structure of the LDL receptor is particularly evident when considering the ligand binding domain. This is a stretch of 292 amino acid residues at the NH_2-terminus of the molecule which is characterized by a string of seven tandemly repeated copies of a sequence of approximately 40 amino acids which is generally referred to as type A repeat. Interestingly, the first of these seven tandem repeats is encoded by exon number 2 (exon 1 encodes the signal sequence necessary for correct targeting of the receptor to the plasma membrane), exon 3 encodes repeat 2, exon 4 encodes repeats 3,4, and 5, exon 5 encodes repeat 6, and exon 6 encodes repeat 7. This modular arrangement has an interesting implication: since the positions of introns in this region of the gene occur in the same reading frame, loss or addition of exons can result in loss or addition of individual modules or blocks of modules without interrupting the reading frame. This would result in molecules with limited, expanded, or simply altered binding ability.

Each one of the seven repeats is characterized by the presence of six cysteine residues at conserved positions. These cysteines have been shown to be involved in the formation of intra-repeat disulfide bonds, resulting in folding of the domain into a rigid structure. The feature that allows this domain of the LDL receptor to interact with its specific ligands is the presence, near the COOH-terminus of each cysteine-rich repeat, of a cluster of negatively charged amino acids. Interaction between the receptor and its two known ligands, apolipoprotein-B and -E, is believed to occur through ionic interactions between these clusters of negative charges on the receptor and complementary clusters of positively charged residues present along the sequence of both apo-B and apo-E (118). More precisely, it has recently been shown that binding of LDL (which contains apo-B) and of ß-migrating very low density lipoprotein (ß-VLDL), which contains multiple copies of apo-E, requires different combinations of repeats (119). LDL binding requires repeats 2 through 7; in contrast, ß-VLDL binding only requires the presence of repeat 5. Thus, different ligands are apparently recognized by the concerted action of different combinations of repeats. The whole binding domain, in summary, seems to have evolved by duplication of a single exon resulting in the production of seven repeats of a single 40-amino-acid sequence. Interestingly, the repeat sequence is strongly homologous to a stretch of 40 amino acids that occurs in the middle of the C9 component of complement (120).

V.A.2. The EGF precursor-like domain

The emblematic feature of this domain, consisting of approximately 400 amino acid residues, is its homology to a portion of the extracellular domain of the membrane-bound precursor for epidermal growth factor. This homology does not extend to the portion of the EGF precursor from which active EGF is obtained, but rather, it involves a part of the EGF precursor which is on the NH_2-terminal side of EGF itself. In particular, three separate regions of the LDL receptor, designated A,B, and C, share with the EGF precursor a type of cysteine-rich repeat, referred to as type B repeat, which is similar to but different from the repeats in the ligand binding domain. These repeats also contain six conserved cysteines but lack the COOH-terminal clusters of negatively charged residues and their cysteine spacing differs from that of the repeats in the binding domain. Repeats A,B, and C are coded for by three of the five exons shared by the genes for the LDL receptor and the EGF precursor. EGF precursor-like repeats have been found in a large number of proteins which, though not functionally related, share one common trait: the ability to mediate protein-protein interactions (reviewed in ref. 121).

Some clues regarding the role of EGF-like repeats in LDL receptor function have recently emerged from two lines of investigations. First, site-directed mutagenetic studies in which repeat A was deleted indicated that the corresponding truncated receptor had reduced LDL binding ability, whereas the binding of ß-VLDL was not affected (122). Similarly, a naturally occurring point mutation in the growth factor-like repeat C results in impaired binding of apo-B (123). Deletion of the complete EGF-like domain, a rather dramatic alteration of the structure of the receptor, results in molecules that have lost the ability to undergo the acid-dependent conformational change that allows the receptor to release bound ligand in endosomes and to recycle back to the plasma membrane (124). Second, EGF-like repeats in the LDL receptor have been shown to contain ß-hydroxylated aspartic acid/asparagine residues (125). It is believed that hydroxylation confers divalent cation-binding properties to the domain. In this context, binding of both LDL and ß-VLDL has been shown to be calcium-dependent (95).

V.A.3. The O-linked sugar domain

This domain, entirely extracellular and immediately preceding the membrane-spanning region, consists of a stretch of 58 amino acids that contains 18 serine or threonine residues and which is encoded by a single exon. Most, if not all, of the 18 hydroxylated amino acids are glycosylated. The function of this particular domain is not clear; it can be deleted without obvious effects on receptor function (126).

V.A.4. The trans-membrane domain

This domain is composed of approximately 20 amino acid residues characterized by their hydrophobic nature. Comparison of the amino acid sequences of the human and bovine LDL receptors has revealed that this domain is relatively poorly conserved, suggesting that it performs no specific functions other than anchoring the molecule to the cell membrane.

V.A.5. The intracellular domain

Immediately following the membrane-anchoring domain is a stretch of 50 residues that projects into the cytoplasm. This portion of the molecule is very important for proper LDL receptor function; naturally occurring mutations that result in internalization-defective phenotypes have been mapped to this region (101), suggesting that this domain is involved in targeting LDL receptors to coated pits. Detailed studies have identified a sequence within the first 22 amino acids following the trans-membrane domain which is crucial for internalization (102). The tetrameric sequence NPXY is conserved in LDL receptors from six species and in two other members of the LDL receptor gene family that will be described in the next pages. Site-directed mutagenetic studies showed that replacement of any of these amino acids resulted in markedly reduced internalization (102). NPXY sequences can also be found in the cytoplasmic domain of several cell surface proteins known to be internalized via coated pits (102).

In summary, the LDL receptor is a modular structure formed by building blocks that are shared with numerous other proteins including the EGF precursor, members of the blood clotting system such as factor IX, X, and protein C (117), membrane proteins involved in determination of cell fate in invertebrate organisms (121) and, in particular, two other membrane proteins recently characterized whose properties are strongly supportive of roles in receptor-mediated processes. Such proteins are discussed in the following pages.

V.B. Other members of the LDL receptor family

Recently, two other membrane proteins with multiple copies of type A repeats, as well as of other structural motifs found in the LDL receptor, have been described. One of these, termed glycoprotein 330 (gp 330) is a large membrane protein with similarity to the LDL receptor in at least six separate regions for which amino acid sequence is available (127). This protein has been identified as the target of autoimmunity in Heymann nephritis, a rat model of human membranous glomerulonephritis (128). In this autoimmune pathologic state, circulating anti-gp 330 antibodies bind to the protein at its location in coated pits at the base of the foot processes of glomerular epithelial cells (129). The formation of these antigen-antibody complexes leads to functional damage of the glomerular filter. Although the normal physiological function of gp330 is unknown, its localization to coated pits and endowment with three sequences at its carboxyterminus which are homologous to the signaling sequence required for endocytosis of the LDL receptor (102) suggest that gp 330 is capable of endocytosis. The presence of at least 13 type A repeats is compatible with recognition of apo-B and/or apo-E, but neither has been demonstrated to date.

In contrast to the limited information on gp 330, the complete amino acid sequence of another gene product of the LDL receptor family, termed LDL receptor-related protein (LRP), is known (130). It harbours a total of 31 type A repeats, 20 of which are grouped in 3 individual clusters of no less than 8 repeats each; perhaps not surprisingly, this large (~500 kDa) protein is capable, at least *in vitro*, of interactions with the same apolipoproteins as the LDL receptor (131,132). Type B cysteine-rich repeats are also found in the sequence of LRP and, following the single membrane-spanning domain, a cytoplasmic tail of 100 amino acids contains two copies of the internalization sequence NPXY (102). Also in analogy to the LDL receptor, LRP is a calcium-binding protein (130,131). In spite of its demonstrated widespread tissue-distribution (130), LRP has been hypothesized to function specifically as a liver plasma membrane receptor with specificity for apolipoprotein-E, expected to function in the removal from the plasma of certain apo-E-containing lipoproteins such as chylomicron remnants, spent lipid transport vehicles of intestinal origin. Although initial results seem to support such a role for LRP (132,133), several issues remain to be addressed before LRP can be verified as the chylomicron remnant receptor.

The significance of the presence of several gene products with structural and possibly functional relatedness to the LDL receptor remains to be fully determined. It is possible that, through the presence of additional modules such as type A and B repeats, these proteins have acquired functions not associated with the LDL receptor. Studies performed in this thesis in order to address this question, as well as other recent studies from other laboratories, will be discussed in Chapters VI and VIII .

VI. SCOPE OF THE THESIS

Because of the lack of direct knowledge of the properties of vitellogenin receptors prior to the start of this thesis, and with the expectation that such knowledge would enlarge our understanding of the process of receptor-mediated endocytosis, the experiments presented in this thesis were designed with the following specific aims:

1) to identify and characterize chicken oocyte VTG receptors;
2) to investigate the molecular basis of their interaction with VTG;
3) to verify the applicability of the findings obtained in chickens to other oviparous species;

4) to evaluate the role of the VTG receptors in the overall process of vitellogenesis in chickens;

5) to evaluate the findings obtained from the study of vitellogenesis in the larger context of other lipoprotein receptors belonging to the LDL receptor gene family.

FIGURE I 1

HEPATIC-OVARIAN RELATIONSHIP IN A VITELLOGENIC FEMALE VERTEBRATE

Vitellogenin is synthesized in the liver in response to estrogen, carried by the circulation to the ovary, and incorporated into developing oocytes. After its internalization, vitellogenin is cleaved to yield lipovitellin and phosvitin.

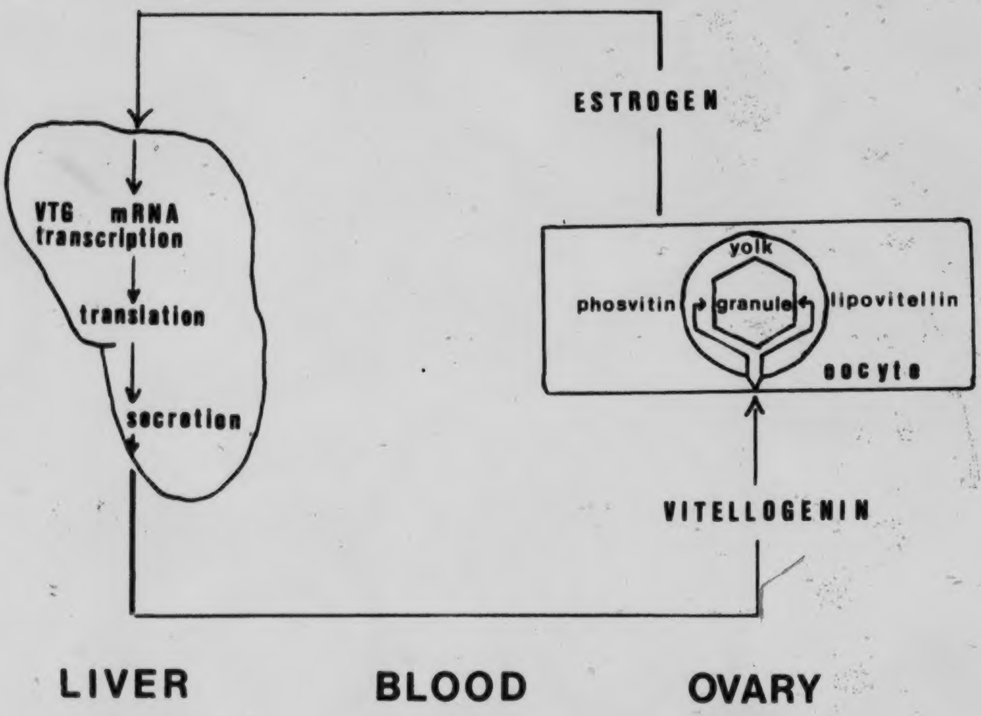
ESTROGEN
VTG mRNA transcription
translation
secretion
yolk
phosvitin
granule
lipovitellin
oocyte
VITELLOGENIN
LIVER
BLOOD
OVARY

FIGURE I 2

SCHEMATIC REPRESENTATION OF THE LDL RECEPTOR

Shown are the five domains of the mature protein described in the text.

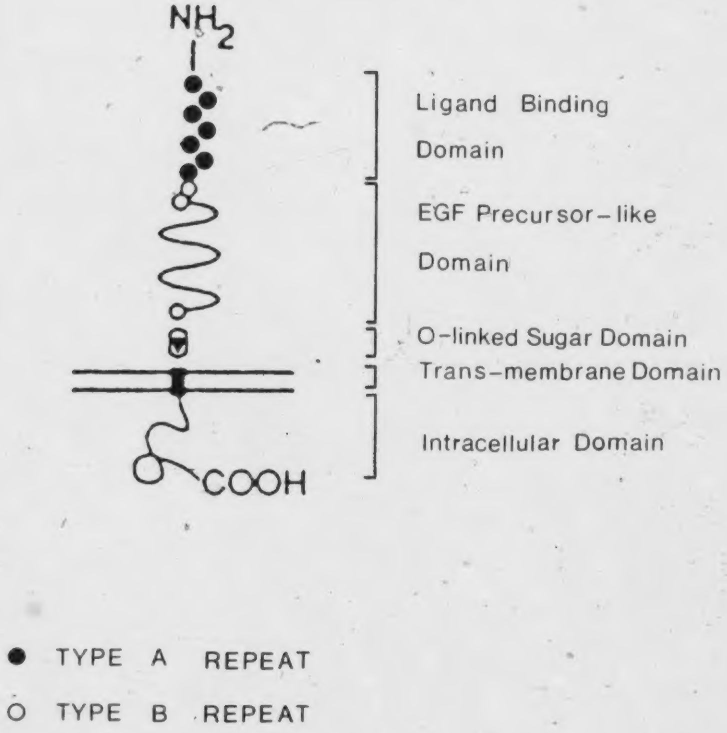
NH2
Ligand Binding Domain
EGF Precursor-like Domain
O-linked Sugar Domain
Trans-membrane Domain
Intracellular Domain
COOH
● TYPE A REPEAT
○ TYPE B REPEAT

TABLE I 1

PROPERTIES OF VERTEBRATE VITELLOGENINS

Data combined from Refs. (58) and (134).

PROPERTIES OF VITELLOGENINS

Molecular weight	440,000 - 480,000
Density (g/ml)	>1.21
Total lipids (% by weight)	12
Polar lipids (% of total lipids)	75
Neutral lipids (% of total lipids)	25
Total P (% by weight)	1.5-2.0
Carbohydrate (% by weight)	1.0 - 1.5
Calcium (% by weight)	1.0 -1.5

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Chapter II

SOLUBILIZATION AND CHARACTERIZATION OF THE CHICKEN OOCYTE VITELLOGENIN RECEPTOR[1]

INTRODUCTION

This work describes the biochemical characterization of the chicken oocyte plasma membrane receptor for vitellogenin. The receptor was extracted from oocyte membranes with the nonionic detergent octyl-β-D-glucoside and visualized by ligand blotting with ^{125}I-VTG as a protein with an apparent molecular weight of 96,000, under nonreducing conditions. It exhibited relatively low affinity for native chicken VTG ($K_d \sim 2 \times 10^{-7}$ M) and was unable to bind VTG with reductively methylated lysine residues or phosvitin. Polyclonal antibodies to the 96-kDa protein inhibited VTG binding to the receptor and were able to precipitate functional VTG-receptor activity from oocyte membrane detergent extracts with concomitant removal of the 96-kDa protein. Antibodies directed against the mammalian receptor for low density lipoprotein showed cross-reactivity with the chicken oocyte VTG receptor, raising the possibility that lipoprotein receptors in birds are structurally related to those in mammalian species.

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MATERIALS AND METHODS

Materials

DEAE-cellulose DE 52 was obtained from Whatman; protein A-Sepharose CL4B and Sephadex G-25 PD 10 columns were from Pharmacia Fine Chemicals; Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was from Pierce Chemical Co.; octyl-β-D glucoside, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, phosvitin, and bovine serum albumin (BSA) were from Sigma; molecular weight standards were from BRL; nitrocellulose paper BA 85 was from Schleicher and Schuell, Keene, NH; Nuflow cellulose acetate membrane filters N25/45 UP were from Oxoid Ltd.; Swinnex W/O filtration units (Cat. No. SXOOO25OO) were from Millipore; sodium (^{125}I) iodide (11-17 mCi/μg) was from Edmonton Radiopharmaceutical Centre, Edmonton, Alberta; suramin (sodium salt) was from FBH Pharmaceuticals, NY. Other materials were obtained from previously reported sources (1).

Animals and Diets

White Leghorn layers (8-18 months old) were obtained from a local poultry farm and maintained on a layer mash, with a light period of 12 hr. Adult female New Zealand rabbits were used for the production of polyclonal antibodies.

Purification of Vitellogenin

Vitellogenin was purified from laying hen plasma as described in (2) with some modifications. All operations were carried out at 4 °C. About 10 ml of blood was drawn from the wing vein and collected into ice-cold tubes containing such quantities of reagents to have the final following concentrations: 16 mM sodium citrate, 1 mM PMSF, 2 μM leupeptin, and 2.5 μg/ml aprotinin. Plasma was then prepared by centrifugation at 3,000 x g for 15 min. After recovery, the plasma was centrifuged at 200,000 x g for 2 hr using Beckman quick seal tubes. After this time, the high speed subnatant fluid that had separated from the low density fraction was recovered and immediately subjected to anion-exchange chromatography. The DEAE-cellulose matrix was prepared as described (3) and packed into 32 x 1.9 cm colmns (bed volume approximately 60 ml). Columns were first rinsed with 200 ml of a buffer containing 100 mM sodium citrate (pH 5.5), 2 mM $CaCl_2$, 1 mM PMSF, and 0.2 % Triton X-100, and subsequentely equilibrated with 200 ml of a buffer containing 50 mM sodium citrate (pH 5.5), 2mM $CaCl_2$, and 1 mM PMSF (buffer A). All buffers used in the purification of vitellogenin contained 2 mM $CaCl_2$ because the presence of this divalent cation seemed to improve the stability of the protein. Ten to fifteen ml of the high speed subnatant fluid was applied to the equilibrated columns, followed by washing with 200 ml of a solution containing 100 mM sodium citrate (pH 5.5), 2mM $CaCl_2$, and 1 mM PMSF (buffer B). One hundred ml of a linear salt gradient of from 0 to 300 mM NaCl in 100 mM sodium citrate (pH 5.5), 2 mM $CaCl_2$, and 1 mM PMSF was then applied to the column in order to elute vitellogenin. After elution, VTG-containing fractions, as determined by monitoring UV light absorbance, were pooled and dialyzed at 4 ^{o}C against two quick changes of a buffer containing 150 mM NaCl, 2mM $CaCl_2$, and 5 mM Tris-HCl (pH 7.8) (buffer C). After dialysis, leupeptin (2 μM), aprotinin (2.5 μg/ml), and PMSF (1 mM) were added to reach the indicated final concentrations and the protein was stored at -70 ^{o}C in aliquotted fractions. Protein concentrations were determined as described by Lowry *et al.* (4), using bovine serum albumin as the standard. Phosvitin concentrations were determined as described in (5).

Protein Iodination and Modification

Vitellogenin and phosvitin were radiolabeled with ^{125}I by the Iodogen method (6). A typical iodination reaction was carried out with 200 μg of Iodogen adsorbed to a glass scintillation vial to which 1.0-2.0 mg of protein were added in buffer C. Sodium [^{125}I] iodide was separated from free ^{125}I by gel filtration on a Sephadex G-25 column previously equilibrated with buffer C. After gel filtration, the labeled protein was dialyzed against several changes of buffer C until almost no radioactivity could be detected in the buffer. ^{125}I-labeled VTG was stored at 4 °C and used within one week.

Methyl-VTG was prepared by treatment with formaldehyde plus sodium borohydride (7). Typically, one volume of protein (ca. 2 mg/ml) was diluted to 1.5 volumes with 0.3 M sodium borate pH 9.0. At 4 °C, 1 mg of $NaBH_4$ was added at 0 time, followed by 1 µl additions of a 37% solution of HCHO at 6 min intervals. After 30 min, 1 mg of $NaBH_4$ was again added and further 1 µl additions of HCHO every 6 min were performed. Sixty min from initiation, the reaction was stopped by dialyzing the reaction mixture against buffer C for at least 18 hr. At the end of the dialysis, methyl-VTG was recovered and its electrophoretic purity tested by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of Oocyte Membranes

All operations were performed at 4 °C. Ovarian follicles (3-15 mm in diameter) were rapidly transferred to buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM PMSF, and 2 µM leupeptin (buffer D). Theca externa and theca interna were removed by dissection and the yolk extruded through an incision. The remaining material, consisting of basal lamina, granulosa cell layer, perivitelline layer, and oocyte plasma membrane, was rinsed with buffer D until the wash fluid was free of yolk. The membranes were minced with scissors and subjected to homogenization in 10 ml of buffer D per g wet weight with a Polytron homogenizer for 30 sec at setting 5, followed by two periods of 20 sec each at setting 8. Large debris was removed by centrifugation at 5,000 x g for 5 min, and the resulting supernatant subjected to centrifugation at 100,000 x g for 1 hr. The membrane pellets were suspended in buffer D by aspiration through a 22-gauge needle, and resedimented by centrifugation at 100,000 x g for 1 hr; this was repeated once. The washed membrane pellets were quickly frozen in liquid nitrogen and stored at - 70 °C for up to 3 months before use.

Solubilization of Chicken Oocyte Vitellogenin Receptors

All operations were carried out at 4 °C. In a typical solubilization experiment, membrane pellets obtained from the ovary of one chicken (weight of follicles, 7-10 g) were suspended in 1.5 ml of buffer containing 250 mM Tris-Maleate (pH 6.0), 2 mM $CaCl_2$, 1 mM PMSF, and 2 μM leupeptin by aspiration through a 22-gauge needle. The suspension was sonicated twice for 20 sec (Sonifier model W 185, Heat System Ultrasonics, Inc.) with a microprobe at setting 6. Reagents were then added to adjust the suspension to a final volume of 3 ml containing the following components: 3-5 mg of protein/ml, 125 mM Tris-Maleate (pH 6.0), 2 mM $CaCl_2$, 160 mM NaCl, 0.5 mM PMSF, 1 μM leupeptin, and 36 mM octyl-β-D glucoside. The suspension was stirred at 4 °C for 10 min and undissolved material was removed by centrifugation at 100,000 x g for 1 hr. The clear supernatant, designated octylglucoside extract, was either quickly frozen in liquid nitrogen and stored at -70 °C for up to 1 month or used immediately.

Electrophoresis, Transfer to Nitrocellulose, and Blotting Procedures

One-dimensional electrophoresis was performed on 4.5-18% polyacrylamide gradient slab gels (16 x 12 x 0.15 cm) containing 0.1% (w/v) SDS according to the method of Laemmli (8). Typically, each sample (2 to 50 μl) was adjusted to 50 μl with water, after which was added 50 μl of 125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, and 2% (w/v) SDS (O'Farrell's buffer). Samples were then adjusted to a final concentration of 0.1% (w/v) bromphenol blue. Under reducing conditions, samples contained 50 mM dithiothreitol and were heated to 90 °C for 5 min prior to loading.

Nonreduced samples were not heated and not treated with dithiothreitol. Electrophoresis was conducted at 10 °C at either 35 mA/slab gel for ca. 5 hr or at 60 V/slab gel in overnight runs. Gels were calibrated with the following molecular weight standards: myosin, 200,000; phosphorylase b, 97,000; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 26,000; β-lactoglobulin, 18,000; lysozyme, 14,000. At the end of the electrophoretic runs, gels were either stained with Coomassie Blue or transferred to nitrocellulose. For electrophoretic transfer of proteins from SDS slab gels onto nitrocellulose, the gel was placed on wet nitrocellulose and Whatman 3MM paper and Bio-Rad Scotch Brite 3M pads were placed on each side. This "sandwich" was inserted into a Trans-Blot Cell apparatus (Bio-Rad), and the chamber was filled with electrode buffer consisting of 192 mM glycine, 25 mM Tris-base, and 20% (v/v) methanol. Electrophoresis was carried out at 200 mA for at least 8 hr at 10 °C with the anode facing the nitrocellulose side of the sandwich (9). After transfer, the nitrocellulose replicas were used in either ligand- or immunoblotting. For ligand blotting experiments (10) nonreduced samples were applied to SDS-polyacrylamide gels. Nitrocellulose replicas were incubated in 10-20 ml of a buffer containing 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM $CaCl_2$, and 5% non-fat dry milk as blocking agent (buffer E). After incubating 2 hr at room temperature, the incubation mixture received the correct ligand at the concentrations and specific radioactivities indicated in the Figure Legends. Incubations were for 90 min on a rocking platform and after this time nitrocellulose strips were washed with buffer E as follows: two rapid washes (a few seconds) followed by two washes for 10 min each. The same steps were repeated with buffer E without dry milk. In immunoblotting experiments, the same protocol was followed as for ligand blotting, except that the incubation buffer contained 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05 % Triton X-100, and 5% dry milk. Visualization of bound antibodies was accomplished by incubation with ^{125}I-labeled protein A. After blotting, the nitrocellulose strips were dried for about 45 min and autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 films at room temperature for the time indicated in the Figure Legends.

Filtration Assay for Determination of ^{125}I-VTG Binding to Oocyte Membrane Extracts

The concentration of octylglucoside in aliquots of the soluble extracts was adjusted to below its critical micellar concentration by addition of 7 volumes of buffer containing 50 mM Tris-Maleate (pH 6.0), 2mM $CaCl_2$ following a procedure established by Schneider *et al.* (11). VTG-binding sites were collected by centrifugation of the diluted extracts at 100,000 x g for 60 min at 4 °C. The precipitate, designated precipitated octylglucoside extract, was resuspended by aspiration through a 22-gauge needle in buffer containing 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM $CaCl_2$ (buffer F) and used for measurement of VTG-receptor activity. The standard assay mixture (pH 8.0) contained in a volume of 100 µl: 12.5 mM Tris-HCl, 25 mM NaCl, 2 mM $CaCl_2$, 16 mg/ml bovine serum albumin, the indicated amount of protein of precipitated octylglucoside extract, and the indicated concentrations of ^{125}I-VTG (see Figure Legends). Incubation was for 90 min at room temperature and free ^{125}I-VTG was separated from receptor-bound ^{125}I-VTG by filtration as described (11). Duplicate aliquots (80 µl) of each reaction mixture were applied to 25-mm cellulose acetate membrane filters as follows. Prior to use, filters were soaked for at least 1 hr at room temperature in buffer G (buffer F containing 1 mg/ml BSA). Each filter was then placed on a stainless steel holder connected to a vacuum line and washed once with 3 ml of buffer G. Three ml of buffer G was then added to the filter holder without suction and an 80-µl aliquot of the sample was layered on top of the buffer. Suction was instantly applied, after which the filter was washed three times under suction with buffer G (3 ml per wash). The filter was then transferred to a glass tube for radioactivity measurement in a Beckman Gamma 5500 Counter. It was absolutely necessary to filter all the VTG solutions used in these experiments prior to starting the incubation since this protein tends to form insoluble aggregates that can interfere with the assay by clogging the filters and giving high background levels. Pre-filtration was performed with Swinnex filter holders from Millipore and cellulose acetate filters were pre-soaked in buffer F containing 40 mg/ml BSA. Filtered solutions were stored at 4 °C and not used for more than two days. Nonspecific binding represents the amount of ^{125}I-VTG retained by the filters when incubations were performed in the presence of suramin. Specific binding was calculated by subtracting the value for nonspecific binding from the value for total binding.

Immunological Procedures

Antisera against the 96-kDa protein were raised in adult female New Zealand white rabbits by subcutaneous injection of antigen prepared in the following way. The VTG-binding protein, as visualized by ligand blotting, was cut out from a 4.5-18% SDS-polyacrylamide gradient slab gel after electrophoresis under nonreducing conditions. Protein was electroeluted according to the procedure described by Hunkapiller *et al.* (12) and used for immunization. Rabbits received a first injection of antigen in Freund's complete adjuvant and successive booster injections at one week intervals using incomplete adjuvant. Four weeks later, animals were bled from the ear vein and sera tested for the presence of reacting antibody by immunoblotting. IgG fractions were purified from sera on columns of protein A-Sepharose CL4B that were equilibrated with 100 mM sodium phosphate (pH 7.0). The IgG was eluted with a solution containing 1 M acetic acid and 100 mM glycine (pH 2.5), after which the pH was raised immediately by addition of 1M potassium phosphate (dibasic). The IgG fractions were dialyzed against a buffer containing 10 mM potassium phosphate and 150 mM NaCl at pH 7.4 (phosphate-buffered saline) and stored at -70 °C at a concentration of 2 mg/ml. In immunoprecipitation experiments, octylglucoside extract aliquots were incubated with the appropriate antibody fractions in phosphate-buffered saline solutions containing 36 mM octylglucoside according to the conditions described in the legend to Fig. II 10.

RESULTS

Purification and Radiolabeling of Chicken Vitellogenin

VTG was isolated from plasma of laying hens using the method described in the "Materials and Methods" section. In particular, the presence of calcium ions throughout the purification scheme and of the protease inhibitors, leupeptin and aprotinin, resulted in preparations in which breakdown products of VTG were consistently absent upon storage at 4 °C for 5-7 days after isolation. Only fresh preparations that were free of VTG breakdown products were used in the experiments described in this report. When attempts were made to radiolabel VTG with ^{125}I, extensive fragmentation of the protein was initially observed. However, with the Iodogen method and keeping the specific radioactivity below 500 cpm/ng, ^{125}I-VTG free of breakdown products was obtained. Fig. II 1 shows the results of SDS-polyacrylamide gel electrophoresis of a typical VTG preparation (lane D), and also demonstrates that after radiolabeling with ^{125}I vitellogenin remained intact (lanes A and C). The preparations of unlabeled and radiolabeled VTG presented in Fig. II 1 were used in all the experiments described herein.

Binding of ^{125}I-VTG to Oocyte Membrane Octylglucoside Extracts

Octylglucoside extracts of oocyte membranes were analyzed for their ability to bind ^{125}I-VTG by a solid-phase filtration assay (11). Binding reached identical maximum levels at 4, 23, and 37 °C after 40 min, showed a pH optimum of 8.0, and was linear with respect to oocyte membrane protein up to 0.6 mg/ml (George, R., and Schneider, W.J., unpublished observations). Under the standard conditions described in the "Materials and Methods" section, binding of ^{125}I-VTG was saturable and showed relatively low affinity (Fig. II 2A). Furthermore, the saturable component of radiolabeled VTG binding was abolished in the presence of 5 mM suramin, leaving only a nonspecific, linear component. Suramin is a polysulfated, polycyclic hydrocarbon that has been shown to inhibit the binding of VTG to its receptor from oocytes of the insect, *Locusta migratoria* (13), and also inhibits the binding of human LDL (6), apoprotein E-containing lipoproteins (14), and platelet-derived growth factor (15) to their respective receptors. Fig. II 3 shows the chemical structure of suramin. Scatchard analysis (16) of the binding data gave a linear plot indicating a single binding site on oocyte membranes for VTG (Fig. II 2B). The apparent equilibrium dissociation constant (K_d) was calculated as ca. 2×10^{-7} M (96 µg/ml) and a maximum of 13 µg of radiolabeled VTG were bound per mg of protein of precipitated octylglucoside extract.

Identification and Characterization of the Oocyte VTG Receptor

Fig. II 4 shows the VTG-receptor protein of the chicken oocyte as visualized by ligand blotting. Oocyte membranes were solubilized with octylglucoside and aliquots of the detergent extracts were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions, followed by transfer to nitrocellulose paper. Nitrocellulose replicas of the gels were then incubated with ^{125}I-VTG. Lane A shows that the VTG-receptor protein has an apparent molecular weight of 96,000 under nonreducing conditions. The presence of a 50-fold molar excess of unlabeled VTG in the incubation medium resulted in a dramatic reduction of ^{125}I-VTG binding to this protein (lane B). In contrast, no effect on the binding of radiolabeled VTG to the 96-kDa protein was observed in the presence of a 50-fold molar excess of VTG that had been chemically modified by methylation of lysine residues (lane C). This reduced ability of reductively methylated VTG to bind to the 96-kDa protein indicates that lysine residues play an important role in ligand-receptor interactions. When electrophoresis prior to transfer to nitrocellulose was performed in the presence of sulfhydryl-reducing agents, binding of radiolabeled VTG to its receptor did not take place, suggesting that intact disulfide bonds are required for biological activity of the receptor (not shown, but see chapter IV, Fig. IV 2).

The ligand blotting data were supported by parallel solid-phase filtration binding studies: binding of ^{125}I-VTG to precipitated octylglucoside extracts was competitively inhibited by unlabeled VTG but was not significantly affected by methylated VTG (Fig. II 5). The lack of competition by methylated VTG was not due to breakdown of the protein during its chemical modification, since the derivatized VTG appeared intact upon inspection by SDS-PAGE (Fig. II 6).

The ligand specificity of the chicken oocyte VTG receptor was further analyzed by ligand blotting (Fig. II 7). Binding of radiolabeled VTG to the 96-kDa protein was totally inhibited by the presence of 5 mM suramin in the incubation mixture (lane 4), in agreement with the solid-phase binding data presented in Fig. II 2. As much as a 780-fold molar excess of unlabeled phosvitin failed to inhibit the binding of labeled VTG (lane 3). In contrast, only a 50-fold molar excess of unlabeled VTG almost completely abolished receptor binding of ^{125}I-VTG (lane 2).

Use of Antibodies Directed Against the 96-kDa Oocyte Membrane Protein

Polyclonal antibodies directed against the 96-kDa protein were prepared and IgG was isolated by chromatography on protein A-Sepharose as described in the "Materials and Methods" section. The IgG fraction, designated anti-OR-IgG, recognized exclusively the VTG receptor as determined by immunoblotting (not shown, but see chapter IV, Fig. IV 4). More importantly, anti-OR-IgG was tested for its ability to inhibit the binding of radiolabeled VTG to its receptor. Fig. II 8 shows that after incubating with increasing concentrations of anti-receptor antibody, the subsequent binding of ^{125}I-VTG was progressively diminished (lanes 1-5). In contrast, a control IgG preparation from nonimmunized rabbit had no effect on the binding (lanes 6-10). To quantify the blotting results, the regions where radiolabed VTG had bound were cut out from the individual nitrocellulose strips and counted for their content of radioactivity. Fig. II 9 shows that at 0.32 mg/ml, the highest concentration tested, anti-OR-IgG reduced the amount of receptor-bound ^{125}I-VTG by 69%, whereas the radioactivity in the bands obtained with control IgG was unchanged over the entire concentration range. Further evidence that this polyclonal antibody is directed towards the functional oocyte VTG receptor came from immunoprecipitation studies (Fig. II 10). After incubating oocyte extracts with anti-OR-IgG, followed by precipitation with protein A-Sepharose, the supernatant showed a large decrease in VTG-binding activity, as demonstrated both by ligand blotting (Fig. II 10, lane D) and the solid-phase binding assay (Table II 1). The binding activity could be recovered by extraction of the protein A-Sepharose pellet (Fig. II 10, lane A). In contrast, none of the binding activity was immunoprecipitated by control IgG, as shown in Fig. II 10, lanes C and F. Interestingly, when immunoprecipitation was performed using a rabbit polyclonal antibody directed against the bovine LDL receptor (17), a small but significant amount of VTG-binding activity was removed from the extract and recovered in the pellet (Fig. II 10, lanes B and E).

DISCUSSION

The results presented in this chapter demonstrate that chicken oocytes possess plasma membrane receptors for interaction with vitellogenin. The receptor protein was solubilized from oocyte membranes by extraction with the nonionic detergent octyl-β-D glucoside. After solubilization, measurable VTG-binding activity could be recovered in a precipitate obtained by decreasing the detergent concentration to below its critical micellar concentration. As previously shown (11), most of the detergent is thereby removed, facilitating the quantification of receptor binding of lipoproteins. The precipitated octylglucoside extract was used in solid-phase filtration assays which showed that binding of radiolabeled VTG to chicken oocyte membranes is specific and saturable. Scatchard analysis revealed a single binding site with a K_d of ca. 2×10^{-7} M (approxima. 96 µg of VTG per ml). Roehrkasten and Ferenz reported a K_d value of 4.2×10^{-8} M for the binding of vitellogenin to solubilized oocyte membrane proteins from the insect, *Locusta migratoria* (18). Woods and Roth reported a K_d value of 2.5×10^{-6} M for the binding of VTG to chicken oocyte membranes (19), in studies where suspensions of membrane fractions were used to determine VTG binding. The maximum specific binding activity of the precipitated octylglucoside extract observed in the present study was ca. 13 µg of VTG per mg of membrane protein; this value is about 10-fold higher than the value reported by Woods and Roth (19).

An interesting observation was that the binding of VTG to its receptor was inhibited by the drug suramin (Fig. II 2A, and Fig. II 7). Previous studies had shown that the binding of various ligands such as LDL (6), platelet-derived growth factor (15), and locust VTG (13) to their respective receptors can be blocked by suramin. In this respect, the chicken oocyte VTG receptor behaves similarly to other receptors involved in receptor-mediated endocytosis.

When the tecnique of ligand blotting was used to identify the VTG-binding protein, the receptor could be visualized as a protein with an apparent molecular weight of 96,000 under nonreducing conditions. If octylglucoside extracts were subjected to electrophoresis in the presence of disulfide-reducing agents, the binding activity was lost, thus suggesting that intrachain disulfide bonds within the receptor molecule are necessary for retention of its biological activity. This behavior is also analogous to that of the receptors for certain other lipoproteins such as LDL (6) and apolipoprotein-E-containing particles (14).

Previous experimental data indicated that the locust VTG receptor apparently recognizes certain positive charges on the VTG molecule (20); modification of lysine residues reduced the binding of VTG to oocyte binding sites (18). For this reason, reductive methylation of the lysine residues on chicken VTG was performed and the modified VTG (meVTG) was tested for its ability to inhibit the binding of ^{125}I-VTG to its receptor. Both solid-phase filtration assays and ligand blotting experiments indicated that meVTG could not compete with radiolabeled VTG for binding to the 96-kDa oocyte membrane protein (Fig. II 4, and Fig. II 5), while unmodified VTG abolished such binding effectively. The possibility that this observation was due to degradation of VTG during the methylation reaction is highly unlikely, because a comparison by SDS-PAGE of native and methylated VTG showed only a very small change in the electrophoretic purity of the modified protein (Fig. II 6). Therefore, the results suggest that lysine residues may be essential for the interaction of VTG with its receptor. A similar situation was reported for the binding of human LDL to its receptor (7).

The ligand specificity of the oocyte VTG receptor was analyzed by ligand blotting. Excess unlabeled phosvitin, one of the intracellular processing products of endocytosed VTG, failed to block the binding of VTG to its receptor, even when present in very high molar excess (Fig. II 7). This, together with experiments that failed to show any binding of ^{125}I-PV to oocyte membrane proteins in ligand blotting experiments at various pH values or in the solid-phase binding assay (Stifani, S., and Schneider, W.J., unpublished observations), seems to rule out the possibility that the phosvitin portion of the VTG molecule contains the binding region recognized by the VTG receptor. Such finding is in contrast with previous reports that claimed that PV can bind to the same receptor as VTG (19), but is in agreement with the observation that the phosvitin domain is the least conserved among vertebrate VTGs and is absent from invertebrate

VTGs (21) and thus is likely not involved in events leading to internalization by oocytes. It should also be noted that the studies showing binding of PV to the same receptor as intact VTG were performed at pH 6.0. In the present study, no binding of either VTG or PV was observed at pH 6.0. The possibility that the other portion of VTG, namely lipovitellin, is recognized by the VTG receptor was not tested since lipovitellins are not sufficiently soluble to afford binding studies (they form the insoluble portion of the egg yolk).

Since the ligand blotting experiments allowed identification of the position where the VTG-binding protein migrated on SDS-polyacrylamide gels, it was possible to cut out the region of the gel containing the protein, elute it from the gel and use it for immunization of rabbits. The resulting polyclonal antibody was used for further characterization of the 96-kDa protein. First, this antibody fraction was capable of blocking the binding of VTG to the 96-kDa membrane protein immobilized on nitrocellulose (Figs. II 8 and II 9). Second, the VTG-binding activity was precipitated from detergent extracts with the anti-96-kDa protein IgG, but not with control IgG (Fig. II 10, and Table II 1). Finally, the finding that polyclonal IgG directed against the bovine LDL receptor cross-reacts to some extent with the VTG receptor raises the interesting possibility that avian VTG receptors may share structural features with mammalian lipoprotein receptors.

FIGURE II 1

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHICKEN VITELLOGENIN.

Chicken VTG was purified and radiolabeled as described in "Materials and Methods". Electrophoresis was performed on a 4.5-18% SDS-polyacrylamide gradient gel under reducing conditions. Lane A: autoradiogram of ^{125}I-VTG; 0.75 µg of protein were loaded (specific activity, 254 cpm/ng). This portion of the gel was dried and autoradiographed for 4 hr. Lane B: molecular weight standards; from top to bottom, myosin (200,000), phosphorylase b (97,000), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (26,000), β-lactoglobulin (18,000), and lysozyme (14,000). Lane C: photograph of lane A. Lane D: 1.5 µg of unlabeled VTG. Proteins were stained with Coomassie Blue. The marks indicate the top of the separating gel.

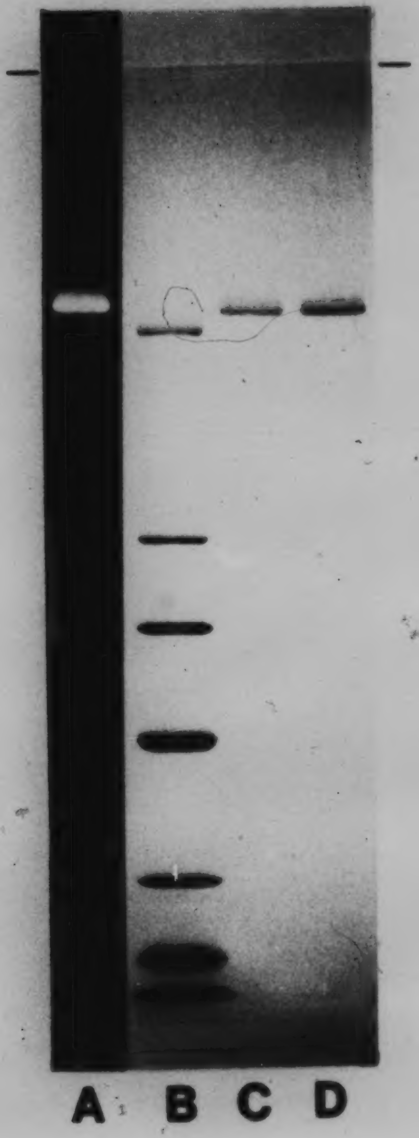

A
B
C
D

FIGURE II 2

BINDING OF ^{125}I-VTG TO PRECIPITATED OCTYLGLUCOSIDE EXTRACTS FROM OOCYTE MEMBRANES

In A, each assay tube contained the standard assay mixture (100 µl) with 33 µg of protein of precipitated octylglucoside extract and the indicated concentrations of ^{125}I-VTG (254 cpm/ng) in the absence (■) or presence (△) of 5 mM suramin. High affinity binding (□) was determined by subtracting nonspecific binding (△) from total binding (■). Each data point represents the average of duplicate incubations. B, Scatchard plot of the high affinity binding data. The ratio bound/free is the amount of bound labeled VTG (µg of protein/ml) divided by the amount of unbound protein in the reaction mixture (µg of protein/ml).

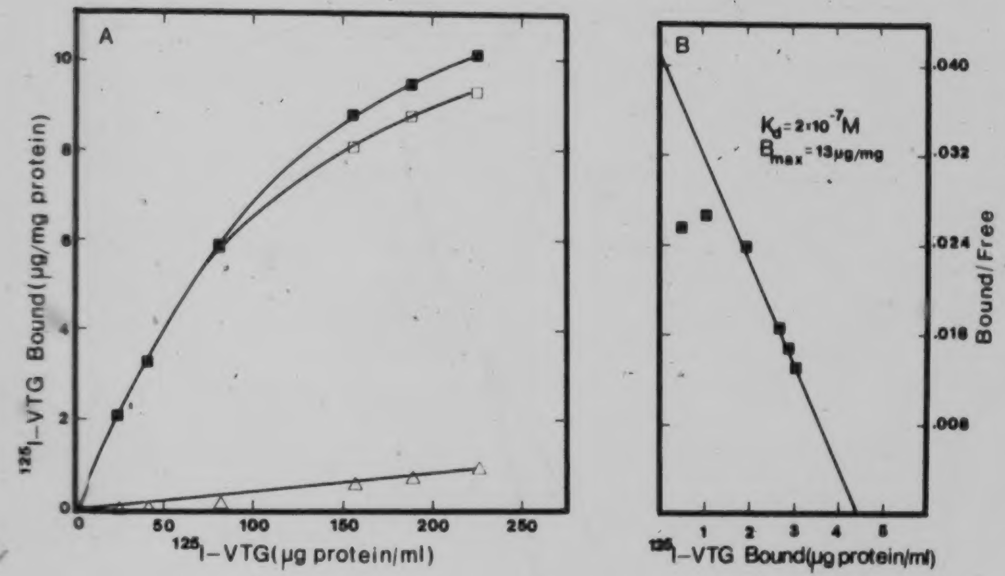
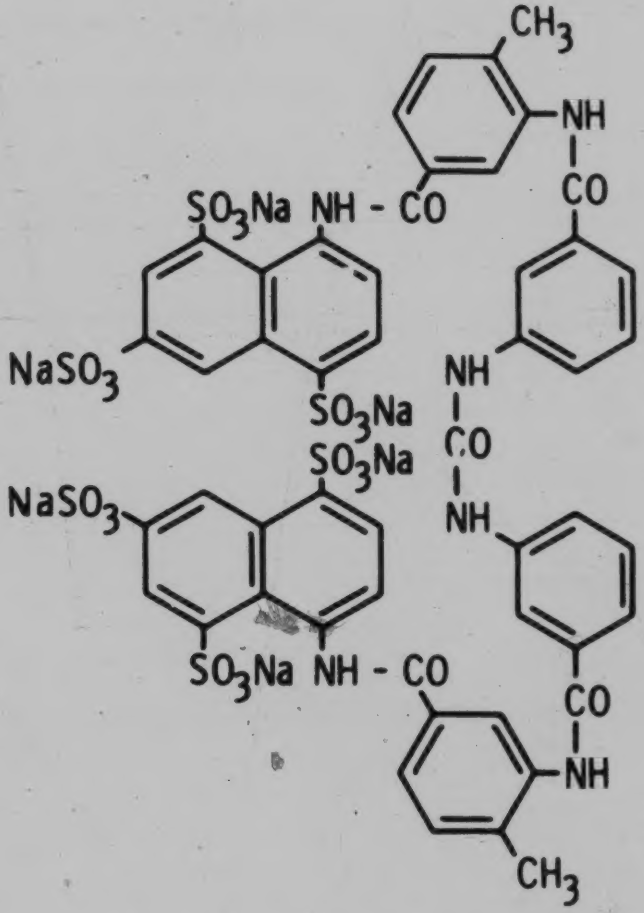

A
0
2
4
6
8
10
^{125}I-VTG Bound (µg/mg protein)
0
50
100
150
200
250
^{125}I-VTG (µg protein/ml)
B
$K_d = 2 \times 10^{-7}$M
$B_{max} = 13$µg/mg
.040
.032
.024
.016
.008
Bound/Free
1
2
3
4
5
^{125}I-VTG Bound (µg protein/ml)

FIGURE II 3

CHEMICAL STRUCTURE OF SURAMIN


CH$_3$
NH
CO
SO$_3$Na
NH - CO
NaSO$_3$
SO$_3$Na
NH
CO
NH
SO$_3$Na
NaSO$_3$
SO$_3$Na
NH - CO
CO
NH
CH$_3$

Suramin

FIGURE II 4

LIGAND BLOTTING OF OOCYTE VTG RECEPTORS

Oocyte membrane octylglucoside extract (35 µg of protein/lane) was subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose. Ligand blotting was performed as described in the "Materials and Methods" section. All strips were incubated in the presence of 2.6 µg of ^{125}I-VTG/ml (254 cpm/ng) with the following additions: lane A, none; lane B, 130 µg of unlabeled VTG/ml; lane C, 130 µg of unlabeled methylated VTG (meVTG)/ml. The positions of migration of molecular weight standards are indicated. Autoradiography was for 12 hr.

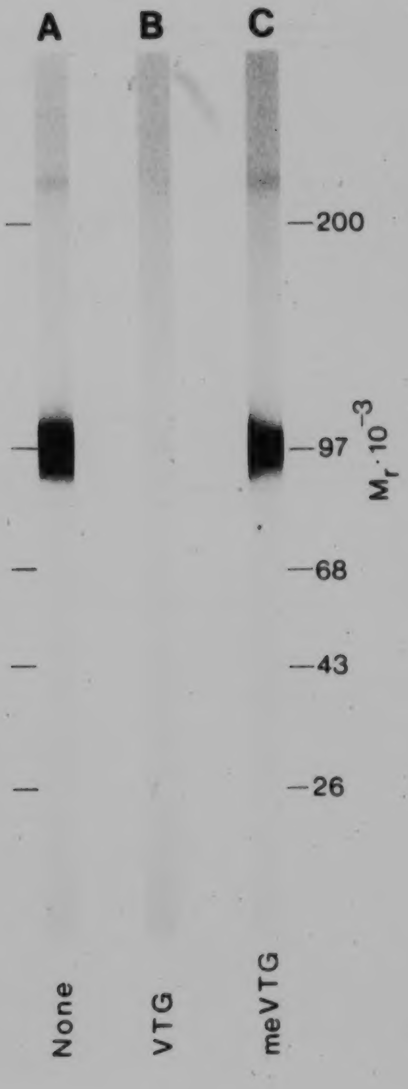

A
B
C
200
97
68
43
26
$M_r \cdot 10^{-3}$
None
VTG
meVTG

FIGURE II 5

COMPETITION OF UNLABELED LIPOPROTEINS FOR BINDING OF ^{125}I-VTG TO OOCYTE VTG RECEPTORS

Each assay tube contained the standard assay mixture (100 µl) with 36 µg of protein of the precipitated octylglucoside extract, 15 µg of ^{125}I-VTG/ml (254 cpm/ng) and the indicated concentrations of unlabeled VTG (▲) or unlabeled methylated VTG (meVTG) (□).

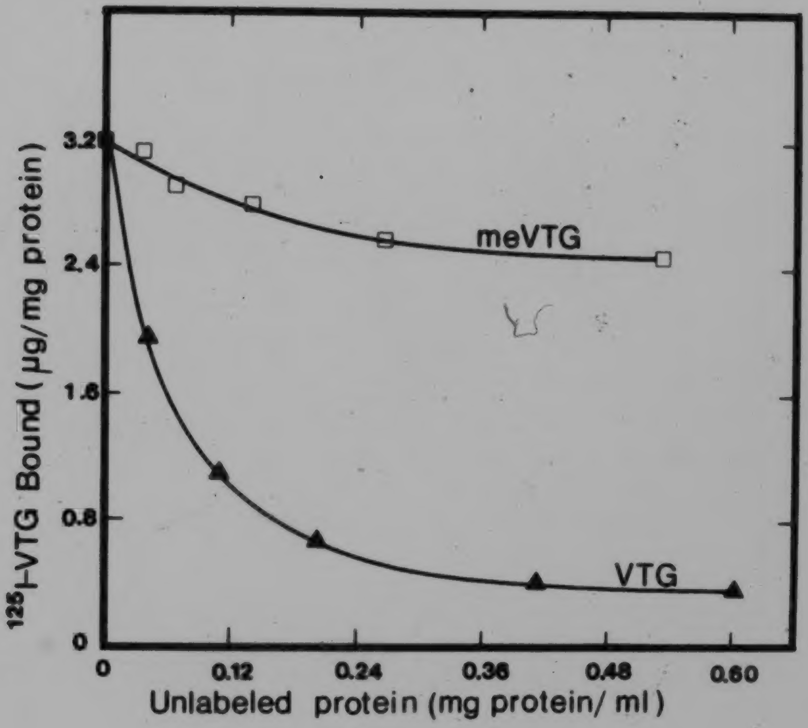

^{125}I-VTG Bound (μg/mg protein)
3.2
2.4
1.6
0.8
0
meVTG
VTG
0
0.12
0.24
0.36
0.48
0.60
Unlabeled protein (mg protein/ ml)

FIGURE II 6

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF REDUCTIVELY METHYLATED VTG

Chicken VTG was purified (lane 1) and subjected to reductive methylation (lane 2) as described in "Materials and Methods". Electrophoresis was performed on a 4.5-18% SDS-polyacrylamide gradient gel under reducing conditions. Lanes 1 and 2 contain 2 µg of protein each. Molecular weight standards (lane 3) are as described in Fig. II 1. Proteins were stained with Coomassie Blue.

VTG
meVTG
STD
1
2
3

FIGURE II 7

SPECIFICITY OF BINDING OF ^{125}I-VTG TO OOCYTE VTG RECEPTORS

Oocyte membrane octylglucoside extract (35 µg of protein/lane) was subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose. All strips were incubated in the presence of 1.75 µg of radiolabeled VTG/ml (127 cpm/ng) with the following additions: lane 1, none; lane 2, 85 µg of unlabeled VTG/ml; lane 3, 100 µg of unlabeled phosvitin/ml; lane 4, 5mM suramin (SUR). The positions of migration of the molecular weight standards are indicated. Autoradiography was for 30 hr.

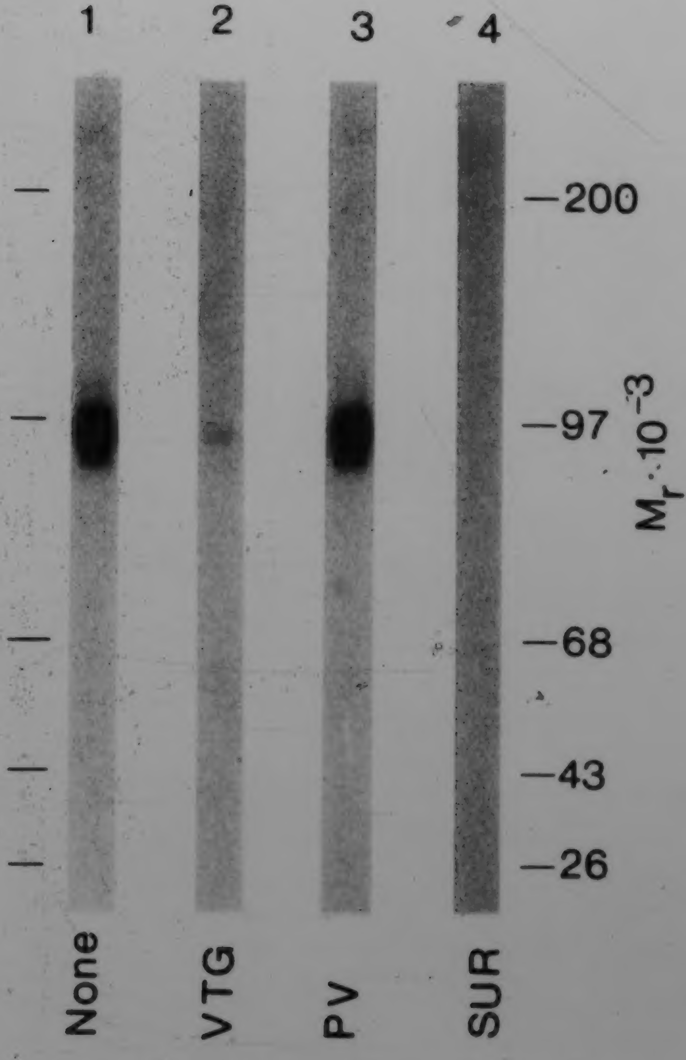

1
2
3
4
—200
—97
—68
—43
—26
$M_r \cdot 10^{-3}$
None
VTG
PV
SUR

FIGURE II 8

INHIBITION OF ^{125}I-VTG BINDING TO OOCYTE VTG RECEPTORS BY ANTI-RECEPTOR ANTIBODY

Oocyte membrane octylglucoside extract (35 μg of protein/lane) was subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose. All strips were incubated in ligand blotting buffer in the presence of either anti-OR-IgG (lanes 2-5) or control IgG (lanes 7-10) at the following concentrations: lanes 2 and 7, 40 μg/ml; lanes 3 and 8, 80 μg/ml; lanes 4 and 9, 160 μg/ml; lanes 5 and 10, 320 μg/ml. The incubations in lanes 1 and 6 contained no IgG. After incubation for 90 min at room temperature, the nitrocellulose strips were extensively washed and then incubated in the presence of 4.9 μg of radiolabeled VTG/ml (85 cpm/ng). The positions of migration of molecular weight standards are indicated. Autoradiography was for 8 hr.

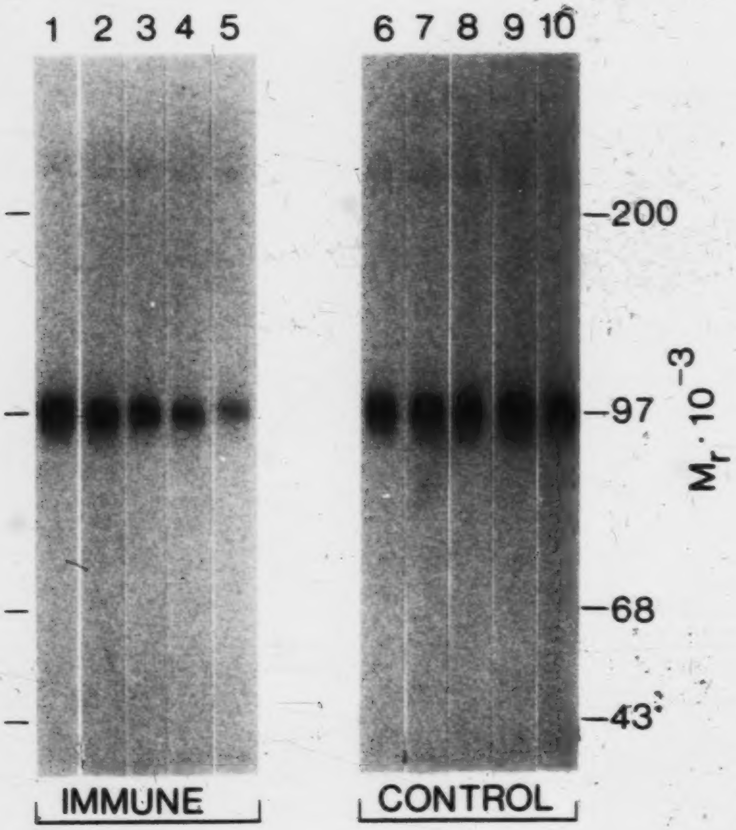

1
2
3
4
5
6
7
8
9
10
200
97
68
43
$M_r \cdot 10^{-3}$
IMMUNE
CONTROL

FIGURE II 9

QUANTIFICATION OF INHIBITION BY ANTI-RECEPTOR ANTIBODY OF ^{125}I-VTG BINDING TO OOCYTE VTG RECEPTORS

The regions of the nitrocellulose strips used in the experiment shown in Fig. II 8 corresponding to receptor-bound ^{125}I-VTG (as indicated by autoradiography) were cut out and counted for radioactivity. Counts were plotted versus the concentration of IgG in the incubation mixture for anti-OR-IgG (□; Imm.) and for control IgG (△; Con.). Data are from one representative experiment; two other experiments gave identical results.

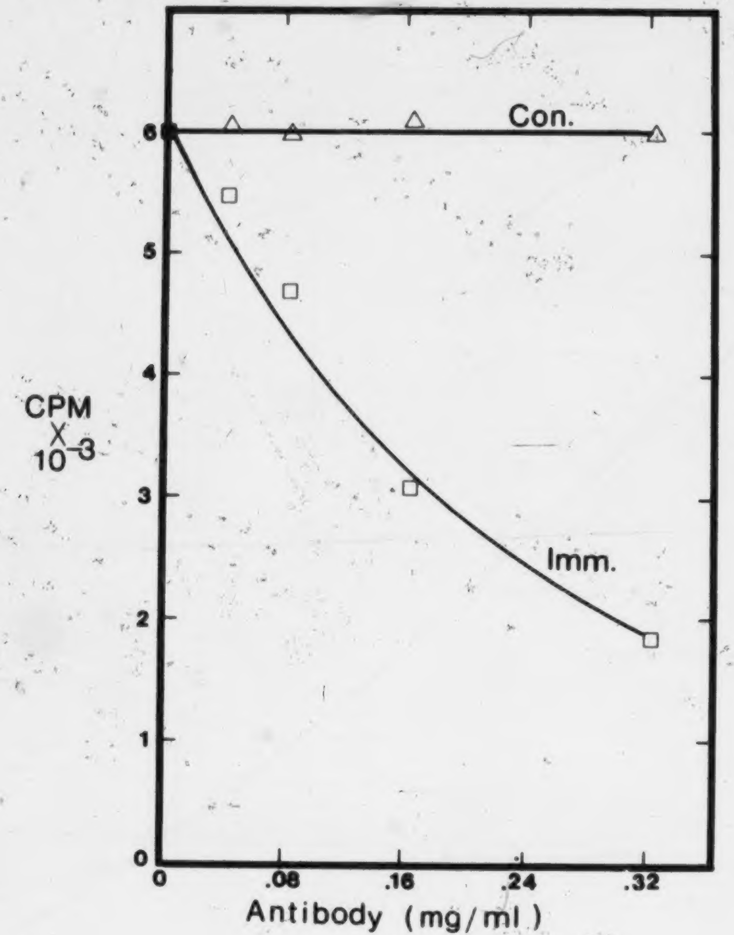

Con.
Imm.
CPM X 10^-3
0
1
2
3
4
5
6
0
.08
.16
.24
.32
Antibody (mg/ml)

FIGURE II 10

IMMUNOPRECIPITATION OF OOCYTE VTG RECEPTORS

Oocyte membrane octylglucoside extract (400 µg of protein in 100 µl) was mixed with 600 µl of buffer containing 10 mM sodium phosphate (pH 7.4), 150 mM NaCl (phosphate-buffered saline) in the presence of 36 mM octylglucoside and 1.75 mg of IgG/ml. Lanes A and D, anti-OR-IgG; lanes B and E, anti-bovine-LDL-receptor-IgG; lanes C and F, control IgG. After incubation for 4 hr at room temperature, 100 µl of a 50% (v/v) suspension of protein A-Sepharose in incubation buffer was added. Following incubation for 2 hr, tubes were centrifuged for 2 min in an Eppendorf microfuge. Both supernatants and precipitates were recovered. Of each supernatant, 60 µl was loaded onto a 4.5-18 % SDS-polyacrylamide gradient gel (lanes D-F). Each precipitate was washed three times with 1 ml of buffer and then incubated with 100 µl of a buffer containing 125 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, and 20% (v/v) glycerol for 15 min at room temperature to release the protein A-Sepharose-bound material. This was recovered in the supernatant following a 90-sec centrifugation step in an Eppendorf microfuge. Portions (50 µl) of each supernatant were loaded onto lanes A-C of the gel. After electrophoresis and transfer to nitrocellulose, ligand blotting was performed with 6 µg of labeled VTG/ml (254 cpm/ng). The positions of migration of molecular weight standards are indicated. Autoradiography was for 16 hr.

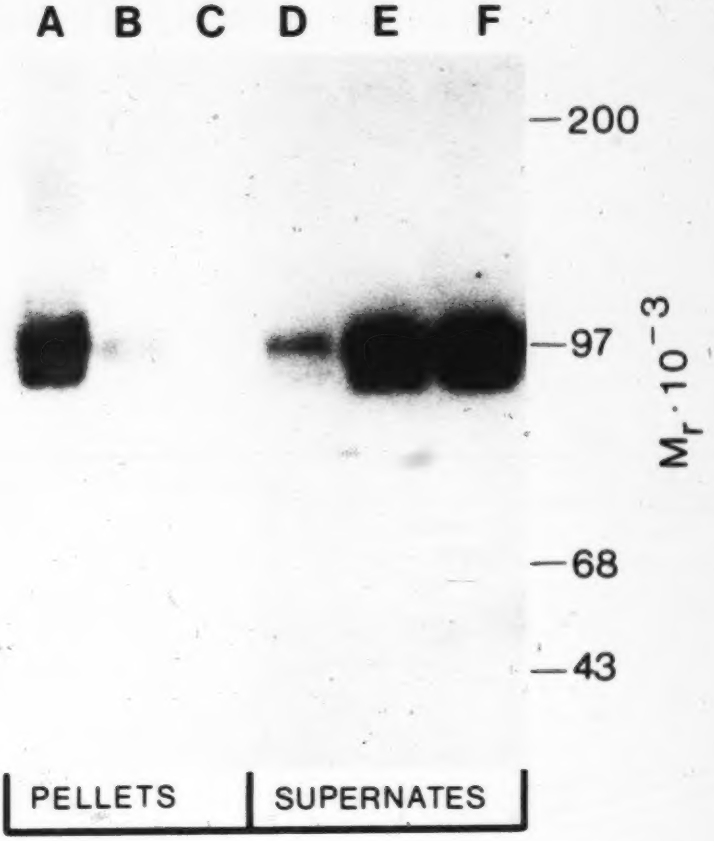

A
B
C
D
E
F
—200
—97
—68
—43
$M_r \cdot 10^{-3}$
PELLETS
SUPERNATES

TABLE II 1

IMMUNOPRECIPITATION OF OOCYTE VTG RECEPTORS

Portions of oocyte membrane octylglucoside extract (150 µl containing 450 µg of protein) were precipitated with the indicated IgG fractions at a concentration of 1.5 mg/ml in a total volume of 800 µl as described in the legend to Fig. II 10. The resulting supernatants (500 µl) were diluted with 3.5 ml of buffer containing 50 mM Tris-maleate (pH 6.0), 2 mM $CaCl_2$, and the precipitated octylglucoside extract collected and resuspended in 500 µl of buffer as described in "Materials and Methods". Binding of ^{125}I-VTG was measured at a concentration of 40 µg/ml (specific radioactivity, 127 cpm/ng) with each tube containing 30 µl of resuspended precipitate, by the solid-phase filtration assay described in "Materials and Methods". Nonspecific binding was determined from incubations containing 5 mM suramin. Each value represents the average of duplicate incubations.

IgG fraction	^{125}I-VTG bound (ng/filter)			^{125}I-VTG bound (% of control)
	Total	Nonspecific	High affinity	
Control	628	14	614	100
Anti-OR	128	19	109	18
Anti-(bovine LDL receptor)	492	21	471	77
None	642	11	631	103

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Chapter III

A SINGLE CHICKEN OOCYTE PLASMA MEMBRANE PROTEIN MEDIATES UPTAKE OF VERY LOW DENSITY LIPOPROTEIN AND VITELLOGENIN[1]

INTRODUCTION

Specific cell-surface receptors mediate the endocytosis of the major yolk components, very low density lipoprotein (VLDL) (1) and vitellogenin (VTG) (2) by growing oocytes of the laying hen. By ligand blotting, we have previously identified the chicken oocyte VLDL receptor as a protein with apparent M_r of 95,000 as determined by SDS-PAGE in the absence of sulfhydryl-reducing agents (1). With the same biochemical tool as used for the identification of the VLDL receptor, namely ligand blotting, a protein to which we assigned an apparent M_r of 96,000 under identical experimental conditions has been shown to be the receptor for VTG on the chicken oocyte plasma membrane (2). Interestingly, a polyclonal rabbit IgG fraction raised against the pure bovine LDL receptor recognized the VLDL receptor (1), and immunoprecipitated VTG-binding activity from chicken oocyte membrane extracts (2). Furthermore, receptor-binding of VTG *and* VLDL was abolished by reductive methylation of lysine residues in both ligands, and exposure of oocyte membrane extracts to sulfhydryl-reducing agents abolished the ability to bind the two ligands (1,2).

[1] A version of this chapter has been published: Stifani, S., Barber, D.L., Nimpf, J., and Schneider, W.J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1955-1959.

These findings of essentially identical apparent M_r's, immunological, and biochemical properties suggested to us a close relationship, if not identity, of the chicken oocyte receptors for VLDL and VTG. In the light of the pivotal role of receptor-mediated endocytosis of yolk proteins in the reproductive effort of the hen, coupled to the oocyte's inability to synthesize yolk proteins (3), it seemed reasonable to us that one and the same chicken oocyte plasma membrane receptor would be responsible for the import of the major yolk lipoproteins, VLDL and VTG. However, it is entirely possible that the receptors are different molecules, since direct evidence for their identity has not been provided.

In the studies reported here, we show that binding activities for the two ligands co-purified on ligand affinity matrices, were inhibited by the same antibody preparations, and the ligands competed with each other for binding to the 95-kDa protein. In addition to these biochemical and immunological lines of evidence for the identity of the vitellogenin receptor with the very low density lipoprotein receptor, genetic proof was obtained. We have shown that the mutant non-laying "restricted ovulator" (R/O) hen carries a defect in the gene responsible for functional expression of the oocyte 95-kDa receptor activity. Here we demonstrate that this single gene defect in the R/O hen has detrimental consequences for the binding not only of very low density lipoprotein, but also of vitellogenin to the 95-kDa receptor normally present in oocytes.

MATERIALS AND METHODS

Materials

We obtained CNBr-activated Sepharose 4B (No. 17-04300-01) from Pharmacia; octyl-ß-D-glucoside, Triton X-100, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and bovine serum albumin from Sigma; and Centricon 30 microconcentrators (No. 4209) from Amicon. All other materials were from previously reported sources (1, 2).

Animals and Diets

White Leghorn hens and roosters were purchased from the Department of Animal Science, The University of Alberta, and maintained as previously described (1, 2). Oocytes were also collected during slaughter by permission of Lilydale Poultry Sales, Edmonton. R/O hens were selected from hatchlings kindly provided by Dr. J. James Bitgood, Poultry Science Department, University of Wisconsin, Madison, and maintained as described in (4).

Preparation of Antibodies

Polyclonal rabbit antibodies against the bovine LDL receptor (1) and the chicken oocyte VTG receptor (2) were raised as described in the indicated references. Polyclonal antibodies against the VLDL-Sepharose affinity-purified receptor were obtained by immunization of adult female New Zealand rabbits as described (4).

Lipoprotein Isolation and Radioiodination

Lipoprotein fractions (1) and vitellogenin (2) were isolated and radiolabeled with ^{125}I as described in the indicated references. In particular, chicken VLDL was obtained from laying hen plasma by ultracentrifugation for 36 hr at 200,000 x g at 4 ^{o}C. The floating lipoprotein fraction was mixed with 150 mM NaCl, 0.2 mM EDTA (pH 7.4), 1 mM PMSF, and 2 μM leupeptin and a second centrifugation step at 200,000 x g for 24 hr was performed. VLDL was recovered from the top of the centrifuge tube. Lipoprotein concentrations are expressed in terms of protein content determined by a modification (6) of the method of Lowry *et al.* (7) using bovine serum albumin as standard.

Preparation and Solubilization of Membrane Fractions.

Oocyte membranes (2) and ovarian membranes from laying hens and R/O hens (4) were prepared and extracted with Triton X-100 as described in the indicated references.

Electrophoresis and Blotting Procedures

One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (8) on 4.5-18% gradient slab gels. Samples were prepared in the absence of dithiothreitol and without heating (nonreducing conditions). Gels were run, calibrated and stained (2) and electrophoretic transfer of proteins to nitrocellulose (2) was performed as described in the indicated references. Ligand blotting was carried out with ^{125}I-labeled VTG or -VLDL as reported (1, 2 and 9). Autoradiography was performed at -70 ^{o}C.

Affinity Chromatography: VTG-Sepharose 4B

Vitellogenin was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (10), using a ratio of 30 mg of VTG/g of dry gel. The VTG-Sepharose 4B was stored at 4°C in a buffer containing 25 mM Tris-HCl (pH 7.8), 50 mM NaCl, 2mM $CaCl_2$, and 0.02% (w/v) NaN_3. Affinity chromatography was carried out at 4°C using columns of 1-cm diameter containing approximately 10 mg of immobilized VTG. Columns were equilibrated with buffer containing 50 mM Tris-HCl (pH 7.8), 4 mM $CaCl_2$, 0.2% Triton X-100 (Buffer A) prior to sample application. Samples, consisting of 200 µl of chicken oocyte membrane Triton X-100 extracts (800 µg of protein), were mixed with 2 volumes of Buffer A and then applied to and recycled over the affinity columns for a total of 2 h. Columns were then washed with 50 bed-volumes of a buffer containing 50 mM Tris-HCl (pH 7.8), 4 mM $CaCl_2$, 0.1% Triton X-

100 (Buffer B) and eluted with 2 bed-volumes of a solution of 0.5 M NH_4OH. The eluted fractions were immediately dialyzed against a buffer containing 25 mM Tris-HCl (pH 7.8), 50 mM NaCl, 2 mM $CaCl_2$ and subsequently concentrated to approximately one fifth of the initial volume using Amicon Centricon 30 microconcentrators. Concentrated material, hereafter referred to as VTG affinity fraction, was immediately applied to SDS-polyacrylamide gels for electrophoresis, transfer to nitrocellulose and ligand blotting as described in the Figure Legends. Control experiments were performed exactly as described above except that bovine serum albumin was covalently coupled to CNBr-activated Sepharose 4B at a ratio of 30 mg of protein/g of dry gel.

RESULTS AND DISCUSSION

When chicken oocyte membrane extracts were analyzed by ligand blotting with radiolabeled VTG and VLDL, protein bands with identical electrophoretic mobility were visualized with both ligands (Fig. III 1). More importantly, excess unlabeled VLDL and VTG inhibited the binding of both radiolabeled yolk precursor proteins, strongly suggesting that the 95-kDa bands visualized by ligand blotting represent the same protein. There was an additional, fainter band migrating above the 200-kDa standard, also observed with both ligands. The intensity of this band varied widely in different preparations of oocyte membrane extracts, and it may represent either an oligomeric form of the receptor, such as described for the mammalian LDL receptor (11), or a protein different from the 95-kDa receptor which exhibits similar properties (see Chapter VI).

In order to further investigate the possibility that the 95-kDa band corresponds to a single protein with dual binding capacity, we pursued our previous observations that polyclonal rabbit IgG fractions raised against the bovine LDL receptor cross-reacted with the chicken oocyte 95-kDa VLDL-binding protein (1,4,12) and could be used to immunoprecipitate the VTG-binding activity in chicken oocyte membrane extracts (2). Furthermore, rabbit antibodies raised against a protein band shown by ligand blotting to bind VTG and subsequently electroeluted from a SDS-polyacrylamide gel effectively inhibited the binding of ^{125}I-labeled VTG to the protein from chicken oocyte membrane assigned an apparent M_r of 96,000 (2). Here, we have obtained another polyclonal rabbit IgG, raised against the fraction obtained after VLDL-Sepharose affinity-chromatography of chicken oocyte membrane extracts (Barber, D.L., and Schneider, W.J., unpublished observations). This IgG fraction inhibited in identical concentration-dependent fashion the binding of ^{125}I-labeled VTG and -VLDL to the 95-kDa band in ligand blots (Fig. III 2). Taken together, these immunological results clearly support our notion that the 95-kDa protein is capable of binding both major yolk precursor proteins.

Our attempts to isolate in pure form the protein with VTG-binding activity by affinity chromatography on VTG-Sepharose in analogy to VLDL-affinity purification have not been successful to date. Our best preparation displays several bands on SDS polyacrylamide gels under nonreducing conditions, with a 95-kDa VTG-binding band constituting approximately 30% of the total protein (Stifani, S., and Schneider, W.J., unpublished observations). Since VTG-affinity chromatography does, however, enrich for the VTG-receptor, we next tested whether VLDL- and VTG-binding activities behaved identically upon chromatography of chicken oocyte membrane extract on VTG-Sepharose. We determined the presence or absence of receptor activity in aliquots of the starting material, unbound, and bound fraction by ligand blotting. As shown in Fig. III 3, VLDL and VTG bound to a 95-kDa band in the crude oocyte extract, as expected (lanes 1 and 4); the unbound fraction was devoid of binding capacity for both ligands (lanes 2 and 5); and the eluted fraction bound both ligands (lanes 3 and 6). We do believe that the VTG receptor *and* the VLDL receptor (if it were different from the former) were bound in specific fashion to the VTG-Sepharose, because Sepharose containing covalently linked bovine serum albumin did not retain the VLDL- nor the VTG-binding activity in nonspecific fashion (lanes 7-12). Thus, the 95-kDa protein binds both VTG and VLDL.

In addition to the strong biochemical evidence for identity of the VLDL receptor with the VTG receptor, we obtained support from a genetic model. We have previously shown that the non-laying "restricted ovulator" (R/O) hen's phenotype is due to the lack of functional oocyte receptors for VLDL (4). Since breeding studies have established that the R/O strain carries a single gene defect (13,14), and since this mutation results in abolition of VLDL receptor activity (4), we tested whether the binding of VTG to R/O ovarian membranes was equally affected. As shown in Fig. III 4, detergent extracts of oocyte and ovarian membranes from laying hens bound ^{125}I-labeled VTG (lanes 1 and 2), whereas detergent extract from R/O ovarian membranes showed a very significant reduction in VTG-binding activity, suggesting that the genetic defect that results in total abolition of VLDL binding also causes a dramatic alteration of the VTG-binding activity of the 95-kDa protein (lane 3).

We believe that these experiments establish that a single plasma membrane protein with an apparent M_r of 95,000 as determined by SDS-PAGE under nonreducing conditions is responsible for the uptake of VLDL and VTG into growing chicken oocytes. We further conclude that the previously described oocyte membrane protein which binds VTG and to which we had assigned an apparent M_r of 96,000 (2) is identical to the receptor described here. The ability of this receptor to recognize two such apparently diverse ligands as apo-B and VTG is surprising at first. However, there are two lines of observations that shed light on this aspect. One group of studies deals with a possible evolutionary link between VTG, apo-B, and lipoprotein lipases (15,16). As shown in these investigations, the VTGs of *Drosophila melanogaster* contain segments with a high degree (up to 40%) of similarity to a region of a large number of lipases; this region of 105 amino acid residues includes a 10-residue putative lipid-binding site located in the N-terminal domain of lipoprotein lipase. More importantly, Baker (15) has identified similarities between the VTGs from *Caenorhabditis elegans*, frog and chicken and human apo-B 100 (mature protein, 4536 residues). Highly significant comparison scores ($P<10^{-25}$) were observed for residues 19-587 of apo-B 100 versus 24-605 of chicken VTG. Such similarities might indicate that apo-B and VTG could bind via common structural elements, possibly to complementary site(s) on the receptor. Unfortunately, the sequence of only 433 amino acid residues at the carboxyl terminus of chicken apo-B (which has approximately the same size as human apo-B) is known (17), and thus, no direct comparison between chicken VTG (1850 residues) and chicken apo-B in the N-terminal region is possible at present. Nevertheless, it is conceivable that a sufficiently similar domain(s) exists on the two ligands through which they might bind to a common site on the receptor.

However, we do ~~not~~ need to postulate that binding of the two ligands occurs to the same site on the receptor. Namely, in reference to the above, the domain on human apo-B that most likely mediates binding to the human LDL receptor is believed to be located in the carboxyl-terminal one-third of the protein (18,19), outside the region of similarity between apo-B and VTG identified by Baker (15). Also, in addition to the ligand blotting experiments (Fig. III 1), solid-phase filtration binding assays (Barber, D.L., and Schneider, W.J., unpublished observations) demonstrated significant cross-competition for receptor binding. However, in these unpublished studies, it appeared that high concentrations of VTG displaced receptor-bound radiolabeled VLDL completely, while VTG was not completely displaced by VLDL. This suggests that cross-competition is due to steric hindrance by ligand binding to closely spaced, but different sites on the receptor. While the detailed characterization of ligand binding functions must await further analysis, it appears entirely feasible that VTG and apo-B bind to separate domains of the 95-kDa protein. Although we have observed in the present study that the R/O phenotype extends from lack of VLDL binding to the 95-kDa oocyte receptor to greatly reduced VTG binding to the same protein, the presence of a residual VTG-binding activity suggests that the two ligands might bind to two separate sites which are differently affected by gene alterations. This possibility is further supported by the other group of findings relevant to the current investigation, which relate to the capacity of the human LDL receptor to recognize apo-E in addition to apo-B (20). The apo-B/E binding domain of the mammalian LDL receptor is located at its N-terminus and consists of 7 homologous cysteine-rich repeats of ~40 residues each (21, 22). Site-directed mutagenetic studies (23) suggested that these repeats are functionally non-equivalent: while the N-terminally located first repeat does not appear to have a role in ligand binding, repeats 2,3,6, and 7 are required for maximal binding of LDL (via apo-B), and repeat 5 is essential for binding of ß-VLDL (via apo-E; cf. Refs. 24, 25). Thus, apo-B and apo-E, which compete with each other for receptor binding, interact with the same general domain, but different substructures thereof. In addition, the monoclonal anti-LDL receptor antibody, IgG-C7 (26) was shown to inhibit LDL binding to the receptor, although it interacts only with the first repeat which is not required for lipoprotein recognition (27). Hence, cross-competition of two ligands, in general, is entirely compatible with their binding to adjacent but not identical sites on the receptor.

We have previously shown that the chicken oocyte 95-kDa protein binds apo-B as well as ß-VLDL (12), and thus most likely apo-E (24). Since chickens do not synthesize apo-E (28), and mammals do not synthesize VTG, it is interesting to speculate that VTG may represent a counterpart to apo-E that has evolved in oviparous species. In this context, it will be of interest to test whether VTG is recognized by LDL receptors of mammalian species; no such studies have been reported to date.

FIGURE III 1

LIGAND BLOTTING OF OOCYTE MEMBRANE PROTEINS

Oocyte membrane Triton extract (20 µg of protein/lane) was subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient slab gel followed by transfer to nitrocellulose and ligand blotting. Strips in lanes A to C were incubated in the presence of 2.8 µg/ml of ^{125}I-VTG (250 cpm/ng) with the following additions: lane A, none; lane B, 140 µg/ml of unlabeled VTG; and lane C, 140 µg/ml of unlabeled VLDL. Strips in lanes D to F were incubated in the presence of 1.7 µg/ml of ^{125}I-VLDL (95 cpm/ng) with the following additions: lane D, none; lane E, 85 µg/ml of unlabeled VLDL; and lane F, 85 µg/ml of unlabeled VTG. The positions of migration of the M_r standards are indicated. Autoradiography was for 14 h.

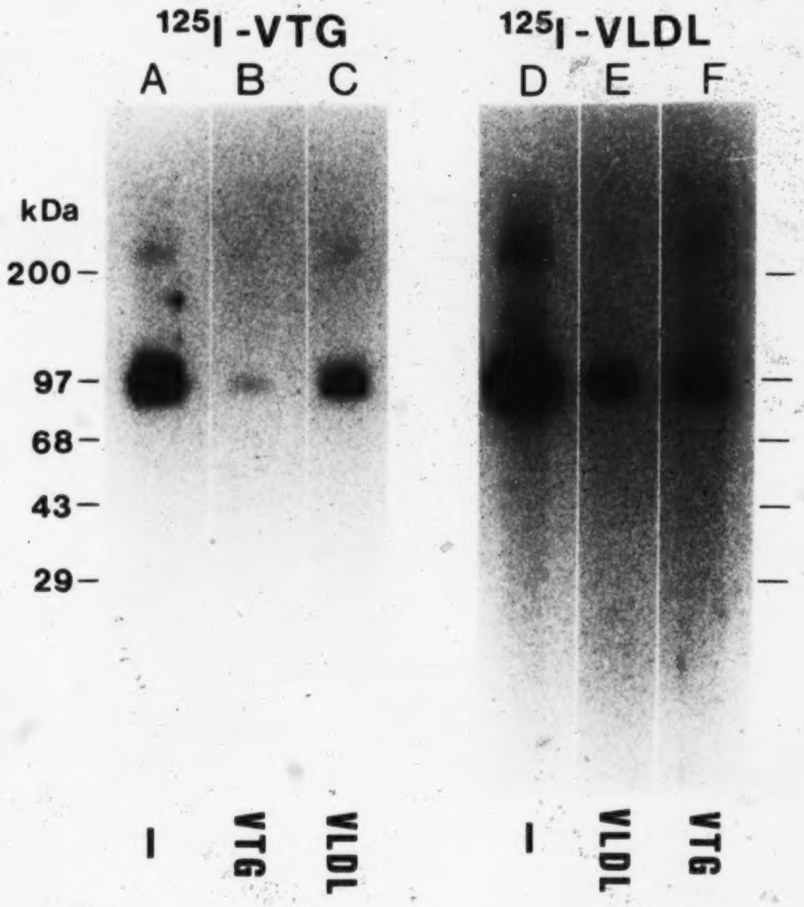

125I-VTG
125I-VLDL
A
B
C
D
E
F
kDa
200
97
68
43
29
-
VTG
VLDL
-
VLDL
VTG

FIGURE III 2

INHIBITION OF ^{125}I-VTG AND ^{125}I-VLDL BINDING TO OOCYTE RECEPTORS BY ANTI-RECEPTOR ANTIBODY

Oocyte membrane Triton extract (30 µg of protein/lane) was subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose as described in the "Materials and Methods" section. Two nitrocellulose strips were incubated, respectively, in the presence of 10 µg/ml of either rabbit anti-VLDL-affinity-fraction IgG (I.) or rabbit nonimmune IgG (C.), followed by ^{125}I-labeled protein A (2.1 µg/ml; 285 cpm/ng). All other strips were first incubated in the presence of either the anti-VLDL-affinity-fraction IgG (lanes 1-4 and 1'-4') or nonimmune IgG (lanes 5-7 and 5'-7') in the following concentrations: lanes 1,5, 1' and 5', none; lanes 2,2',6, and 6', 160 µg/ml; lanes 3, 3', 312 µg/ml; lanes 4,4',7 and 7', 625 µg/ml. After incubation for 90 min at room temperature, nitrocellulose strips were extensively washed and then incubated in the presence of 4.2 µg/ml of ^{125}I-VLDL (95 cpm/ng; lanes 1-7) or 3.5 µg/ml of ^{125}I-VTG (310 cpm/ng; lanes 1'-7'). The positions of migration of M_r standards are indicated. Autoradiography was for 16 hr.

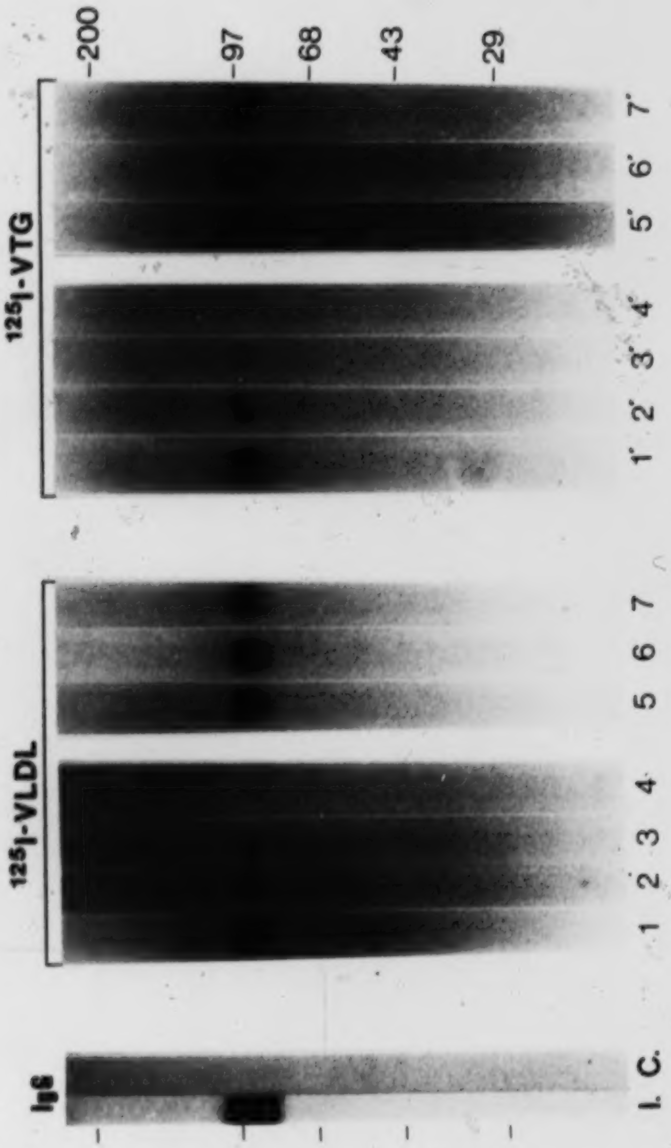

125I-VTG
-200
-97
-68
-43
-29
1'
2'
3'
4'
5'
6'
7'
125I-VLDL
1
2
3
4
5
6
7
IgG
I. C.

FIGURE III 3

LIGAND BLOTS OF OOCYTE MEMBRANE EXTRACTS SUBJECTED TO AFFINITY CHROMATOGRAPHY ON VTG-SEPHAROSE 4B

Oocyte membrane Triton extract (800 µg of protein) was subjected to affinity chromatography on either VTG-Sepharose 4B (lanes 1-6) or bovine serum albumin-Sepharose 4B (lanes 7-12) as described under "Materials and Methods". Aliquots of the starting material (lanes 1,4,7 and 10; 20 µg of protein each), the unbound fraction (lanes 2,5,8 and 11; 20 µg of protein each), and the eluted fraction (lanes 3,6,9 and 12; 3 µg of protein each) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel, followed by transfer to nitrocellulose and ligand blotting with either 2.6 µg/ml of ^{125}I-VTG (310 cpm/ng; lanes 1-3 and 7-9) or 4.2 µg/ml of ^{125}I-VLDL (95 cpm/ng; lanes 4-6 and 10-12). Autoradiography was for 10 hr.

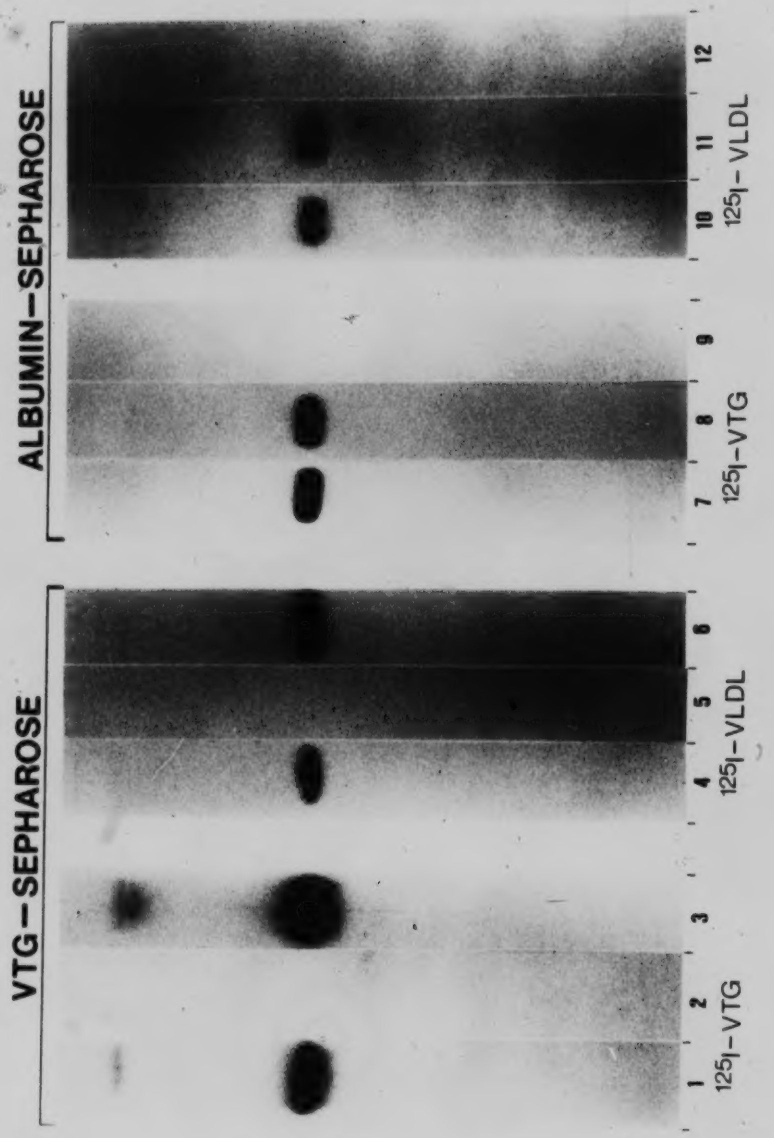
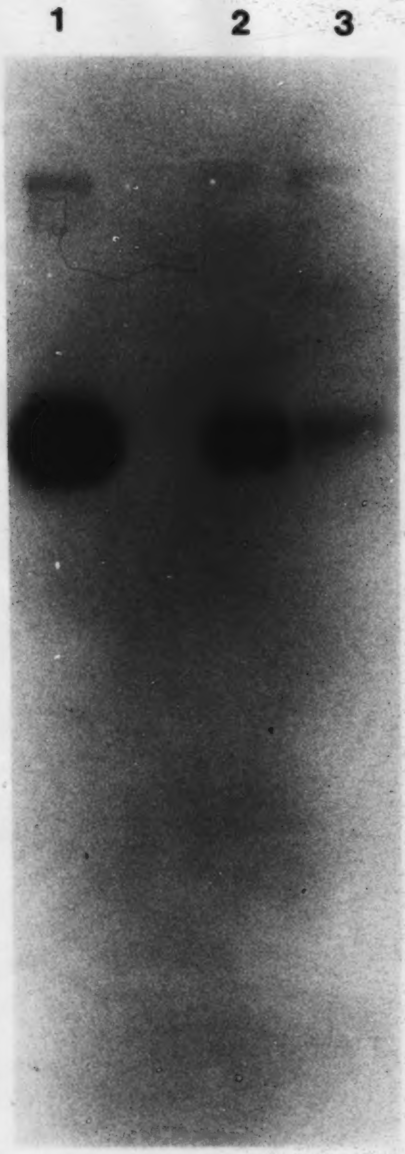

VTG-SEPHAROSE
ALBUMIN-SEPHAROSE
1
2
3
4
5
6
7
8
9
10
11
12
^{125}I-VTG
^{125}I-VLDL
^{125}I-VTG
^{125}I-VLDL

FIGURE III 4

LIGAND BLOTTING OF OVARIAN MEMBRANES

Oocyte membrane Triton extract (20 µg of protein/lane; lane 1) and ovarian membrane Triton extract (40 µg of protein/lane) from either laying hens (lane 2) or R/O hens (lane 3) were subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gel and transfer to nitrocellulose. Incubation of the nitrocellulose replica was in the presence of 2.5 µg/ml of ^{125}I-VTG (310 cpm/ng). Autoradiography was for 16 hr.


1
2
3

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Chapter IV

VITELLOGENESIS IN *XENOPUS LAEVIS* AND CHICKEN: COGNATE LIGANDS AND OOCYTE RECEPTORS[1]

INTRODUCTION

Although the *Xenopus* oocyte is one of the prime experimental systems for expression of foreign genes and their products, the receptor for the main vitellogenic protein, vitellogenin, from this extensively utilized cell has not been identified. Here we have applied ligand and immunoblotting to visualize the *Xenopus laevis* oocyte receptor for vitellogenin as a protein with an apparent M_r of 115,000 in sodium dodecyl sulfate-polyacrylamide gels under nonreducing conditions. The receptor from the amphibian oocyte also recognizes chicken vitellogenin, and *vice versa*; furthermore, the two receptor proteins are immunologically related as revealed by Western blotting with anti-chicken vitellogenin receptor antibodies. In addition, we have begun to address the issue of specificity of these receptors in regards to the VTG domain they recognize. The receptors from both species bind the lipovitellin moiety of vitellogenin, as revealed by ligand blotting with radiolabeled lipovitellin polypeptides as well as by a novel reverse ligand blotting procedure utilizing nitrocellulose-immobilized ligand. Since vitellogenins of chicken and *Xenopus* have been shown to be structurally similar and evolutionarily related (1), it appears that conservation of key structural elements required for efficient vitellogenesis extends from the ligands to their receptors on the oocyte plasma membrane.

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[1] A version of this chapter has been published: Stifani, S., Nimpf, J., and Schneider, W.J., (1990) *J. Biol. Chem.* **265**, 882-888

MATERIALS AND METHODS

Materials

We obtained octyl-ß-D-glucoside, Triton X-100, PMSF, leupeptin, aprotinin, phosvitin (Cat. No. P-1253), bovine serum albumin, human chorionic gonadotropin and Ponceau S from Sigma; nitrocellulose paper BA 85 from Schleicher and Schuell, Keene, NH, U.S.A. Other materials were obtained from previously reported sources (2).

Experimental Animals

Female *Xenopus laevis* animals were kindly donated by Dr. S. Zalik, (Dept. of Zoology, The University of Alberta) and by Dr. N. Milos (Dept. of Anatomy and Cell Biology, The University of Alberta). Animals used for oocyte retrieval had been regularly injected with 800 I.U. of human chorionic gonadotropin over the last 12 months (at 3-months intervals) and had received their last injection 30 days prior to ovarectomy. White Leghorn laying hens were provided by Dr. F. Robinson (Dept. of Animal Sciences, The University of Alberta) and maintained as previously described (2). Adult female New Zealand rabbits were used for the production of polyclonal antibodies.

Protein Isolation and Radioiodination

Xenopus vitellogenin was purified from plasma of female frogs by DEAE-cellulose chromatography (3). After elution, a mixture of protease inhibitors (2) was added prior to storage. Chicken VTG was obtained as previously described (2). Lipovitellin was obtained from the yolk of freshly laid chicken eggs according to the salt fractionation procedure of Bernardi and Cook (4), with the exception that all buffers contained 1 mM PMSF and 2 µM leupeptin. The lipovitellin pellet obtained after centrifugation of the 50 mM $MgSO_4$ fraction (4) was redissolved in an amount of buffer containing 1 M NaCl, 5 mM Tris-HCl (pH 7.8) to give a final protein concentration of 8 - 10 mg/ml. Lipovitellin remained soluble under these conditions and could be stored at 4 °C for more than one month without appreciable degradation. In order to be used in ligand blotting experiments, lipovitellin was first dialyzed for 12 h at 4°C against a buffer containing 500 mM NaCl, 5 mM Tris-HCl (pH 7.8), 0.2% (v/v) Nonidet P-40 and subsequently for 24 h against a buffer containing 250 mM NaCl, 5 mM Tris-HCl (pH 7.8), 0.2% (v/v) Nonidet P-40. After dialysis the protein solution was centrifuged for 5 min at 10,000 x g in order to remove any insoluble material. The supernatant obtained after centrifugation could be stored for up to two weeks at 4°C without appreciable precipitation of lipovitellin. Vitellogenin and lipovitellin preparations were radiolabeled with ^{125}I by the Iodogen method (5) to specific activities not exceeding 500 cpm/ng of protein.

Preparation of Oocyte Membranes and Solubilization of VTG Receptors

All operations were performed at 0-4°C. The ovary was quickly removed from a *Xenopus* female and placed into ice-cold buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 150 mM NaCl, l mM PMSF, 2 µM leupeptin (Buffer A) and the follicles were partially separated from the connective tissue with forceps. Follicles were rinsed with Buffer A and then subjected to homogenization as described (2). Large debris was removed by centrifugation at 5,000 x g for 5 minutes and the resulting supernatant was centrifuged at 100,000 x g for 1 hour. The membrane pellets were washed twice with Buffer A and frozen in liquid N_2 prior to storage at -70°C. Chicken oocyte membranes were prepared exactly as previously reported (2). Oocyte membrane proteins were solubilized in the presence of either 1% Triton X-100 or 36 mM octyl-ß-D-glucoside (2) as indicated in the Figure Legends. In preliminary solubilization experiments, we found higher specific receptor activity when chicken oocyte membranes were extracted with Triton, and frog oocyte membranes with octylglucoside, respectively.

Electrophoresis, Transfer to Nitrocellulose, and Blotting Procedures

One-dimensional electrophoresis was conducted on 4.5 - 18% SDS polyacrylamide gradient slab gels, according to the method of Laemmli (6). Samples were prepared by either heating to 90°C for 5 minutes in the presence of 50 mM dithiothreitol (reducing conditions) or in the absence of dithiothreitol and without heating (nonreducing conditions). Gels were run, calibrated and stained (2) and electrophoretic transfer of proteins to nitrocellulose (7) was performed as described in the indicated references. Ligand and Western blotting were performed as described in (2) except when lipovitellin was used, in which case the incubation buffer consisted of 25 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM $CaCl_2$ and 5% non-fat dry milk. Visualization of bound rabbit IgG in Western blotting experiments was with ^{125}I-protein A. The concentrations and specific radioactivities of the ligands and antibodies used in the incubation mixtures are indicated in the Figure Legends. Autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 film at -70 ^{o}C for the times indicated in the Figure Legends.

Other Methods

^{125}I-labeled protein A was obtained by the Iodogen method (5). The protein content of samples containing no detergent was determined by the method of Lowry et al. (8), and that of samples containing detergents and of vitellogenins and lipovitellins were measured by a modification of the Lowry procedure (9). Antisera against chicken VTG were obtained by immunizing rabbits with pure protein according to (2). IgG fractions were purified from sera on columns of protein A-Sepharose CL-4B (10).

RESULTS AND DISCUSSION

Similarity of Chicken and *Xenopus* Vitellogenins.

Vitellogenins were purified from the plasma of chickens (2) and *Xenopus* (3) as described in the indicated references, and the obtained proteins were analyzed by SDS-polyacrylamide gradient gel electrophoresis. Figure IV 1 shows that pure intact vitellogenins have similar apparent molecular weights (lanes B and C), with chicken VTG exhibiting a slightly slower mobility than *Xenopus* VTG. This is in agreement with the reported amino acid residue number of 1850 for the major chicken VTG, VTG II (11) and of 1807 for the *Xenopus* A2 VTG gene product (12). When Western blotting experiments were performed with a polyclonal rabbit antibody preparation obtained against purified chicken VTG, the chicken and the *Xenopus* VTG molecules were shown to be immunologically related (Fig. IV 1, lanes D and E). The heterogeneity of both VTG preparations, apparent after Western blotting as well as after Coomassie Blue staining of SDS-polyacrylamide gels, is not surprising since chickens (13) produce three and *Xenopus* (14) possibly four vitellogenins, and there may be extensive heterogeneity in regards to phosphorylation among these forms (13).

Identification of *Xenopus* Oocyte VTG Receptors.

In order to test if the close relationship between the vitellogenins of chicken and *Xenopus* is of biological significance for the process of vitellogenesis, we attempted to identify the *Xenopus* oocyte membrane receptor for this ligand. To this end, we applied the technique of ligand blotting, which we had used previously to demonstrate that the chicken oocyte receptor for VTG is a 95-kDa protein (2,15). Thus, oocyte membrane extracts from chicken and *Xenopus* were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing or reducing conditions, the proteins electrophoretically transferred to nitrocellulose and the replicas incubated with ^{125}I-labeled *Xenopus* VTG under appropriate conditions. As shown in Fig. IV 2, lane 4, the radiolabeled frog ligand bound to a single membrane protein from chicken oocytes; this protein has an apparent molecular weight of 95,000 and corresponds to the chicken oocyte membrane receptor for VTG (2). Similarly, only one membrane protein from *Xenopus* oocytes, having an M_r of approximately 115,000 , interacted with ^{125}I-labeled *Xenopus* VTG (Fig. IV 2, lane 3). The binding of radiolabeled VTG to both chicken and *Xenopus* oocyte membrane proteins was totally abolished when membrane proteins were exposed to disulphide-bond reducing agents during gel electrophoresis (Fig. IV 2, lanes 5 and 6). Comparison of the polypeptides present in the oocyte membrane fractions (Fig. IV 2, lanes 1 and 2) with the ligand blots (lanes 3 and 4) demonstrated the high degree of selectivity of VTG binding to the 95-kDa and 115-kDa proteins, respectively. The weaker signal obtained with the oocytes from *Xenopus* in comparison to those from chicken is most likely due to the smaller number of receptors present in the amphibian oocyte preparation. In chicken, the receptor appears to be a major protein component of oocyte membrane extracts (Fig. IV 2, cf. lanes 2 and 4). The loss of binding activity observed following exposure of chicken and *Xenopus* oocyte membrane proteins to disulphide reducing agents (Fig. IV 2, lanes 5 and 6) suggests that intrachain disulphide bonds within the receptor molecules are necessary for retention of their biological activity. Based on the above observations we concluded that the VTG receptor in *Xenopus* oocytes has an apparent M_r of 115,000, and that the chicken oocyte VTG receptor recognizes frog VTG.

Compatibility of VTG Ligands and Oocyte Receptors.

The successful application of ligand blotting to the identification of the Xenopus VTG receptor and the demonstration of the recognition of frog VTG by the chicken oocyte VTG receptor (Fig. IV 2) encouraged us to further test the cross-reactivity of VTG receptors by ligand blotting with radiolabeled chicken VTG. The results of Fig. IV 3 demonstrate that chicken VTG is recognized not only by the 95-kDa chicken oocyte receptor but also by the *Xenopus* oocyte 115- kDa protein (lanes 1 and 2). When a large excess of unlabeled VTG from either species was present in the incubation mixture, the binding of ^{125}I-labeled ligand was greatly diminished (Fig. IV 3, lanes 3-6). The same type of cross-competition was also observed with ^{125}I-*Xenopus* VTG as ligand (Fig. IV 3, lanes 7-12). These results clearly indicate that the two ligands and the two receptors share properties which may be important for their physiological function.

Immunological Cross-Reactivity of VTG Receptors.

The similarity and functional conservation of the chicken and the frog VTG receptors at the level of ligand recognition prompted us to test whether the two proteins were immunologically related. Two different rabbit polyclonal IgG fractions proved most useful for these studies. The first, designated anti-BR IgG, had been raised against the pure bovine low density lipoprotein receptor and had been shown previously to cross-react with the chicken oocyte receptor for VTG (2). The second, designated anti-OR IgG, had been raised against the 95- kDa chicken VTG receptor (2). As shown in Fig. IV 4, both IgG fractions recognized the same bands as those identified by ligand blotting. The observation that polyclonal IgG directed against the bovine low density lipoprotein receptor cross-reacts with *Xenopus* oocyte VTG receptor (Fig.IV 4, lane D) confirms and extends the results obtained with the chicken VTG receptor (Fig. IV 4, lane C, and Ref. 2). Although the molecular basis for such immunological crossreactivity remains to be elucidated, these results suggest that oocyte VTG receptors are not only related amongst oviparous species, but may also share structural features with lipoprotein receptors in mammalian species. Further evidence for a close structural and functional relationship between the oocyte VTG receptors of the chicken and *Xenopus* emerged from an experiment based on our previous observation that the antibody raised against the chicken oocyte receptor inhibited binding of VTG to the chicken receptor (2). Namely, as shown in Fig. IV 5, the same IgG fraction effectively also inhibited VTG binding to the

Xenopus oocyte 115-kDa protein. This finding suggests that binding sites for VTG have been conserved in receptors from two oviparous species.

Specificity of VTG Oocyte Receptors.

After uptake into developing oocytes, vitellogenins are proteolytically processed into two families of yolk proteins, designated lipovitellins and phosvitins (16). Previous studies indicated that neither the binding of chicken VTG (2) nor that of *Xenopus* VTG (17) to their oocyte receptors is mediated by the phosvitin region. Although such findings suggested that the receptor-recognition site is located on the lipovitellin portion, no direct evidence for or against this notion has been presented to date. In order to address this question, we developed a novel approach, reverse ligand blotting, as described below. First, we isolated lipovitellin (LV) from the yolk of freshly laid chicken eggs according to Bernardi and Cook (4). Two types of LV have been identified in chicken yolk granules and have been named α-LV and ß-LV (4,18). Native LVs are dimers (M_r, 400,000) in neutral salt solutions and are reversibly dissociable into monomers at elevated pH. The two avian LVs differ in their subunit composition: while both α- and ß-LVs contain heavy chains (termed LV I) of M_r greater than 100,000 and light chains (termed LV II) of M_r smaller than 40,000 (19), α-LV has been shown to contain additional components of intermediate M_r (20,21). In *Xenopus laevis*, only one type of LV has been described, composed of a large and a small subunit (22). As a result of multiple heterogeneous parent vitellogenin molecules (14,23), each of the subunits can be resolved into three components

When the isolated mixture of chicken egg yolk α- and ß-LV was analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions, a complex array of polypeptides was visualized after staining with Coomassie Blue (Fig IV 6, lane A). A major component migrated with an apparent molecular weight of 120,000-125,000, representing the LV heavy chain (LV I). In addition, a protein band doublet was present at positions corresponding to apparent M_r's of about 105,000 and 100,000, respectively. It is not known at present whether these constitute degradation products of the more abundant 125-kDa polypeptide or reflect a heterogeneous LV population analogous to that described for frog oocytes (23). A major component of M_r 30,000, visualized by Coomassie Blue (see below) was tentatively identified as the light chain (LV II). Other polypeptides with apparent M_r's of 78,000, 41,000 and 38,000 were also present. The electrophoretic band pattern was identical under reducing and non-reducing conditions (Stifani, S., and Schneider, W.J., unpublished observations). Wallace & Morgan (24) reported an almost identical electrophoretic pattern after subjecting total yolk granules to SDS-polyacrylamide gel electrophoresis. However, the presence of a cluster of polypeptides in the M_r range 28,000 - 43,000, shown to be phosvitin(s), added further complexity to their results. We believe that our LV preparation was free of phosvitin contamination based on the following observations. First, the same bands could be visualized whether or not Coomassie Blue staining solutions contained $AlCl_3$, whose inclusion is necessary for the visualization of heavily phosphorylated proteins, such as phosvitin (23). Second, when the polypeptide composition of isolated LV was compared to that of commercially available phosvitin (see Materials), none of the components exhibited identical mobilities (Fig. IV 6, cf. lanes A and B). Third, a rabbit polyclonal IgG fraction obtained against pure, intact chicken VTG cross-reacted with *all* major polypeptides of our LV preparation in immunoblotting experiments, while nonimmune control IgG gave no reaction (Stifani, S., and Schneider, W.J., unpublished observations). Since phosvitins have been reported to be extremely poor immunogens even when part of the intact VTG molecule and normally fail to be recognized by antibodies raised against the parent molecule (16), we believe that our preparation of LV was essentially free of non -α-, non-β-LV polypeptide chains.

The major problem associated with studies of LV is the insolubility of the protein complex in commonly used buffer systems (25). Previous methods for the isolation of LV involved the use of high ionic strength buffers; while this approach permitted successful chromatographic, electrophoretic, and ultracentrifugal analyses (4,18,25), it cannot be applied to investigations of ligand-receptor interactions sensitive to high ionic strength. In order to establish conditions suitable for such studies, the protocol described in "Materials and Methods" was devised to obtain useful LV solutions. We then performed ligand blotting experiments in which LV was tested for its ability to interact with oocyte VTG receptors. Figure IV 7 shows that the binding of ^{125}I-labeled chicken VTG to both the chicken and the frog receptor was abolished not only by excess unlabeled VTG (lanes 3 and 4), but also by excess unlabeled lipovitellin (lanes 5 and 6). As expected, in a control incubation the presence of phosvitin had no effect on the binding of ^{125}I-VTG (lanes 7 and 8). As shown in Fig. IV 8, qualitatively the same results were obtained when ^{125}I-labeled LV was used as ligand. SDS-polyacrylamide gel electrophoresis and autoradiography revealed incorporation of radioiodine into all LV polypeptides (Stifani, S., and Schneider, W.J., unpublished observations). LV bound to both the chicken and the *Xenopus* oocyte receptor (lanes 1 and 2); this binding was specific in that it was effectively reduced by excess unlabeled LV (lanes 3 and 4) or unlabeled VTG (lanes 5 and 6), but it was unaffected by excess unlabeled phosvitin (lanes 7 and 8).

Since we showed that LV contains the domain involved in mediating VTG binding to its receptor, we next attempted to identify the polypeptide chain(s) carrying such domain. However, LV complexes are extremely difficult to disaggregate; exposure to 4 M urea (26) or 6 M guanidinium hydrochloride (22) only results in dissociation of the LV dimers into monomers with no further dissociation into their subunits. In addition, column chromatographic purification of the LV subunits requires prior heating to 100°C in buffer containing 10% SDS and 10 mM dithiothreitol (23), conditions very likely to render the molecules biologically inactive. Since all means required to achieve and maintain dissociation of the LV subunits are incompatible with studies of ligand-receptor interactions, liquid-phase incubation of LV subunit(s) and the oocyte VTG receptor is unfortunately not possible. We have therefore developed an indirect approach to address this question. This consisted in performing reverse ligand blotting experiments in which LV polypeptides were separated on SDS-polyacrylamide gels under nonreducing conditions, followed by transfer to nitrocellulose; the replicas were then incubated with detergent-solubilized chicken oocyte membrane proteins in buffer previously shown to allow ligand-receptor interactions in direct ligand blots. After washing, the VTG receptor molecules bound to LV subunit(s) were visualized by incubation with anti-VTG receptor IgG (anti-OR IgG, cf. Fig. IV 4) followed by incubation with ^{125}I-protein A. As shown in Fig. IV 9, lane 2, the VTG-receptor interacted with LV polypeptides of molecular weight 125,000, 78,000, 41,000 and 30,000. No bands were visualized in control experiments in which the incubation with oocyte membrane detergent extract was omitted or in which nonimmune IgG instead of anti-OR IgG was used (Stifani, S., and Schneider, W.J., unpublished observations). Furthermore, failure to observe any signal at the positions on the nitrocellulose replicas corresponding to polypeptides of the phosvitin preparation (Fig. IV 9, lane 1; compare with Fig. IV 6, lane B) confirmed our notion that phosvitin is not the receptor-binding domain of VTG. Thus, reverse ligand blotting appears to be a useful tool for the identification of receptor-binding domains of macromolecular ligands.

The results of Figs. IV 7 to IV 9 strongly support the notion that LV contains the receptor recognition domain of VTG; however, the exact location within the vitellogenin sequence will require further studies, for two reasons. *First*, the possibility that some of the smaller receptor-binding polypeptides represent degradation products of the 125,000 chain cannot be ruled out. Based on the complete amino acid sequence of the chicken VTG II molecule, van het Schip *et al.* (11) suggested that the heavy (125,000) and the light (30,000) LV subunits are derived, respectively, from the aminoterminal and the carboxyterminal sides of the phosvitin section, which occupies an internal position. However, no direct evidence such as NH_2-terminal amino acid sequence of isolated fragments has been provided to date, except for phosvitin. Unfortunately, such hypothetical model does not help to explain the presence of LV polypeptides of intermediate size nor to locate their original positions within the parent molecule. Hence, it is entirely possible that the 30-kDa LV fragment that is recognized by the VTG receptor may be a degradation product of the aminoterminal heavy chain and thus may not be LV II. *Second,* we do not know the exact pattern of LV chains derived from the minor chicken VTG, VTG I; no sequence information is available for the VTG I molecule, and no models have been proposed to account for the observed yolk fragments it generates. In this context, Wang & Williams (21) concluded from limited proteolysis mapping that VTG II gives rise to polypeptides in both α-LV and ß-LV, while VTG I gives rise to only α-LV polypeptides. Clearly, further investigations into the postendocytic processing of VTG in the chicken oocyte, analogous to studies performed in the insect, *Blatella germanica* (27), and for chicken apolipoprotein B (28) are required.

In conclusion, we believe that our experiments strongly suggest that the 115-kDa protein from *Xenopus* oocytes is the receptor for VTG, and that the binding of this ligand to receptors in chicken and *Xenopus* is mediated by a site located in the lipovitellin moiety, presumably on its heavy chain (LV I). Although we have developed and applied a novel tool to determine the receptor binding domain of complex ligands (reverse ligand blotting), it is the identification of the *Xenopus* oocyte VTG receptor that is particularly encouraging since these studies represent a breakthrough in over two decades of attempts to identify this receptor in a prime experimental system of modern molecular and developmental biology (29). In addition, the results indicate that the homology among vertebrate VTGs is indeed significant in terms of oocyte receptor recognition, and that the similarity between the ligands extends to their receptors.

FIGURE IV 1

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHICKEN AND FROG VTG AND IMMUNOBLOTTING WITH ANTI-CHICKEN VTG ANTIBODY

Xenopus VTG (lanes B, D, and F) and chicken VTG (lanes C, E, and G) were prepared and subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel under reducing conditions as described under "Materials and Methods". A portion of the gel (lanes D to G) was used for Western blotting; proteins were transferred to nitrocellulose and incubated with 10 µg/ml of rabbit anti-chicken-VTG IgG (lanes D and E) or nonimmune IgG (lanes F and G) as described under "Materials and Methods". The bound antibodies were detected by incubating with ^{125}I-labeled protein A (2.1 µg/ml; 285 cpm/ng). Autoradiography of lanes D to G was for 5 h. Lanes A-C were stained with Coomassie Blue. Lane A, M_r standards, from top to bottom: myosin (200,000), phosphorylase B (97,000), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (26,000), ß-lactoglobulin (18,000), and lysozyme (14,000).

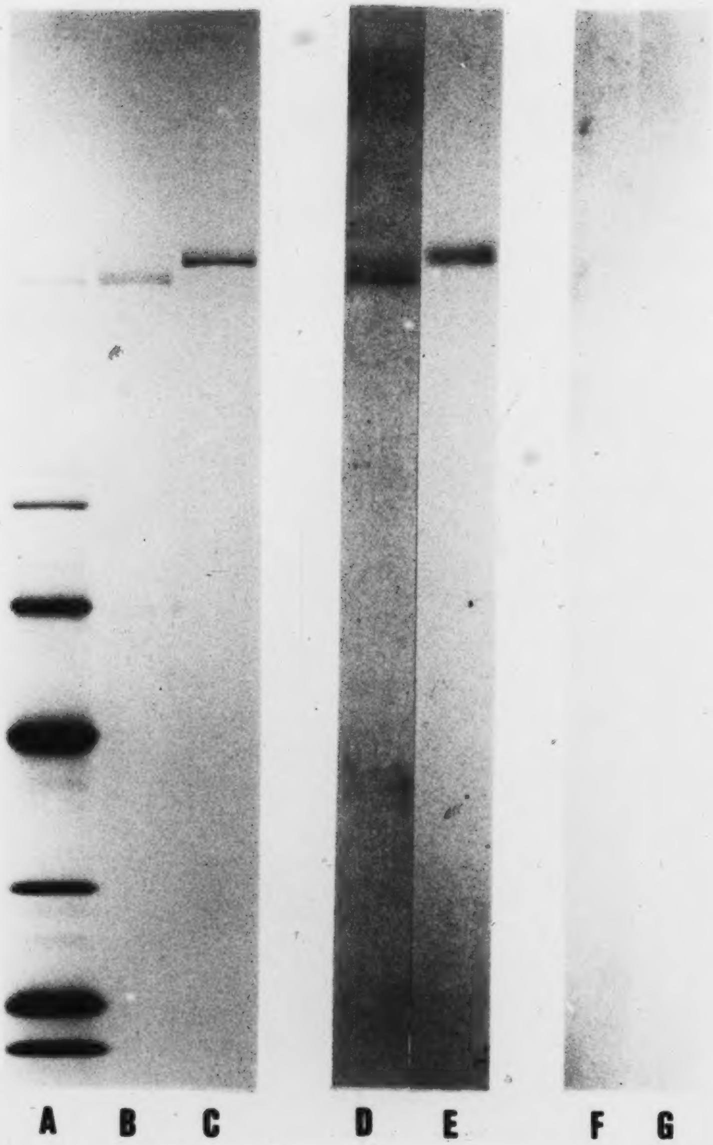

A
B
C
D
E
F
G

FIGURE IV 2

LIGAND BLOTTING OF OOCYTE VTG RECEPTORS UNDER NONREDUCING AND REDUCING CONDITIONS: BINDING OF ^{125}I-*XENOPUS* VTG

Chicken oocyte membrane Triton extract (lanes 2,4,6; 20 µg of protein/lane) and *Xenopus* oocyte membrane octylglucoside extract (lanes 1,3,5; 100 µg of protein/lane) were subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose. Prior to electrophoresis samples in lanes 5 and 6 were heated to 95°C for 5 minutes in the presence of 50 mM dithiothreitol, while samples in lanes 1 to 4 were applied without heating or treatment with dithiothreitol. Lanes 1 and 2 were stained with Ponceau S. Ligand blotting (lanes 3-6) was performed as described under "Materials and Methods". Strips in lanes 3,4 (nonreducing conditions) and in lanes 5,6 (reducing conditions) were incubated with ^{125}I-*Xenopus* VTG (1.0 µg/ml; 497 cpm/ng). The positions of migration of the M_r standards are indicated. Autoradiography (lanes 3 - 6) was for 4 h.

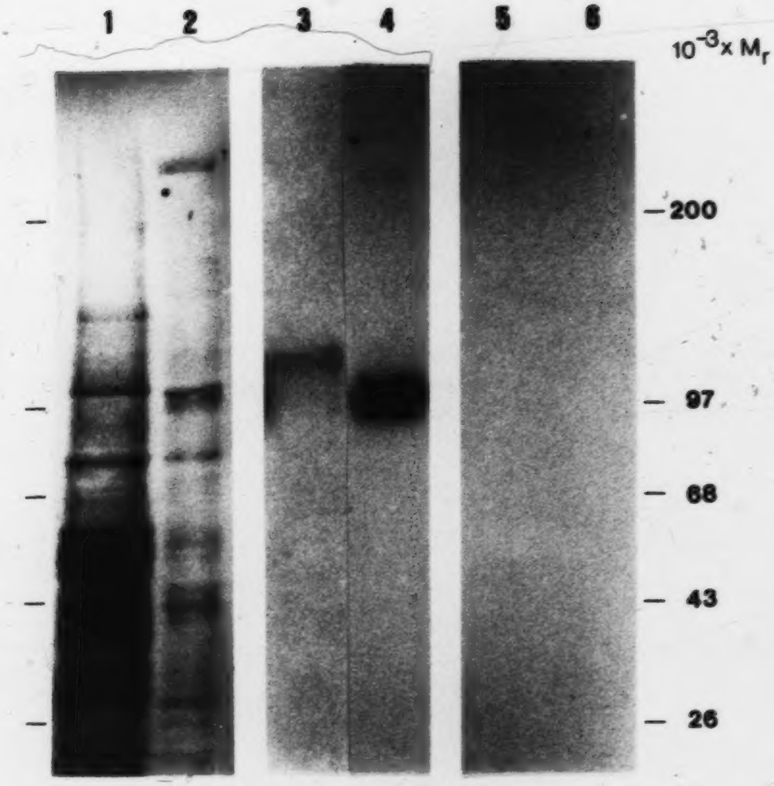

1
2
3
4
5
6
$10^{-3} \times M_r$
200
97
68
43
26

FIGURE IV 3

COMPATIBILITY OF VTG LIGANDS AND OOCYTE RECEPTORS

Chicken oocyte membrane Triton extract (lanes 1,3,5,7,9,11; 20 μg of protein/lane) and *Xenopus* oocyte membrane octylglucoside extract (lanes 2,4,6,8,10,12; 100 μg of protein/lane) were subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and ligand blotting as described under "Materials and Methods". In panel (a), all strips were incubated in buffer containing 2.8 μg/ml of ^{125}I-chicken VTG (212 cpm/ng) with the following additions: lanes 1,2, none; lanes 3,4, 113 μg/ml of unlabeled chicken VTG (ChVTG); lanes 5,6, 113 μg/ml of unlabeled *Xenopus* VTG (XVTG). Autoradiography was for 6 h. In panel (b), all strips were incubated in buffer containing 1.0 μg/ml of ^{125}I-*Xenopus* VTG (497 cpm/ng) with the following additions: lanes 7, 8, none; lanes 9, 10, 50 μg/ml of unlabeled chicken VTG (ChVTG); and lanes 11, 12, 50 μg/ml of unlabeled *Xenopus* VTG (XVTG). Autoradiography was for 24 h. The positions of migration of the M_r standards are indicated.

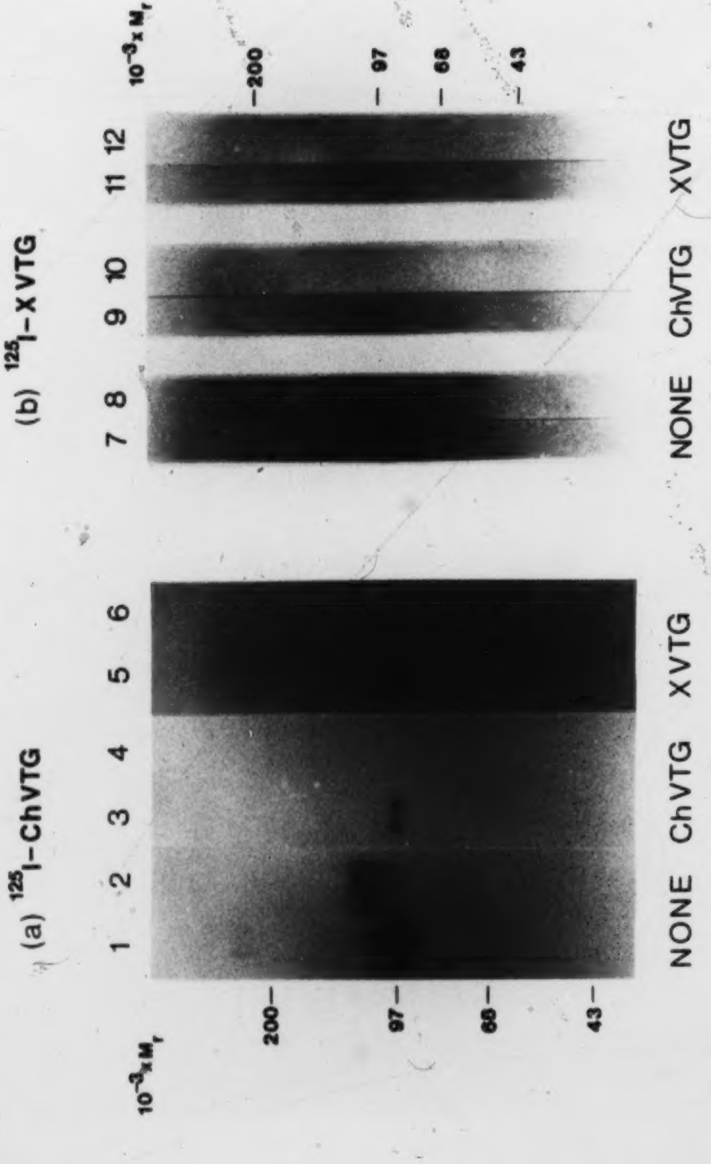

(a) 125I-ChVTG
(b) 125I-X VTG
1 2 3 4 5 6
7 8 9 10 11 12
NONE ChVTG XVTG
NONE ChVTG XVTG
10-3 x Mr
200
97
68
43
10-3 x Mr
200
97
68
43

FIGURE IV 4

IMMUNOBLOTTING OF OOCYTE VTG RECEPTORS

Chicken oocyte membrane Triton extract (lanes A, C; 20 µg of protein/lane) and *Xenopus* oocyte membrane octylglucoside extract (lanes B, D; 100 µg of protein/lane) were subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and Western blotting as described under "Materials and Methods". In lanes A and B, incubation was with 10 µg /ml of rabbit anti-chicken-VTG-receptor IgG (anti-OR IgG) and in lanes C and D with 20 µg/ml of rabbit anti-bovine-low density lipoprotein-receptor IgG (anti-BR IgG), followed by ^{125}I-labeled protein A (2.1 µg/ml; 285 cpm/ng). The positions of migration of the M_r standards are indicated. Autoradiography was for 17 h.

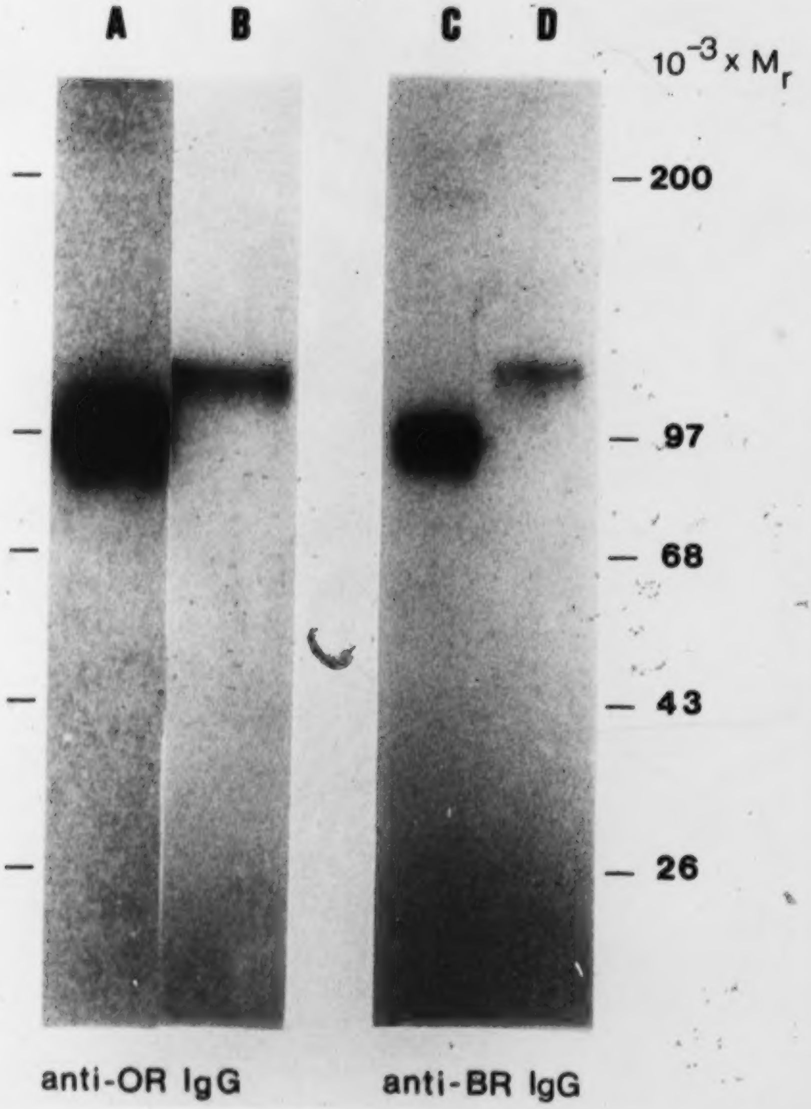

A
B
C
D
$10^{-3} \times M_r$
200
97
68
43
26
anti-OR IgG
anti-BR IgG

FIGURE IV 5

INHIBITION OF ^{125}-VTG BINDING TO *XENOPUS* OOCYTE VTG RECEPTORS BY ANTI-CHICKEN VTG RECEPTOR ANTIBODY

Xenopus oocyte membrane octylglucoside extract (100 µg of protein/lane) was fractionated on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose. All strips were incubated in the presence of either the anti-chicken-VTG receptor IgG (anti-OR IgG; lanes 1-5) or nonimmune IgG (lanes 6-10), in the following concentrations: Lanes 1 and 6, none; lanes 2 and 7, 150 µg/ml; lanes 3 and 8, 300 µg/ml; lanes 4 and 9, 600 µg/ml; and lanes 5 and 10, 1000 µg/ml. After incubation for 90 minutes at room temperature, the strips were extensively washed with buffer and then incubated in 10 ml of buffer containing 2.0 µg/ml of ^{125}I-chicken VTG (212 cpm/ng) as described under "Materials and Methods". The positions of migration of M_r standards are indicated. Autoradiography was for 9 h.

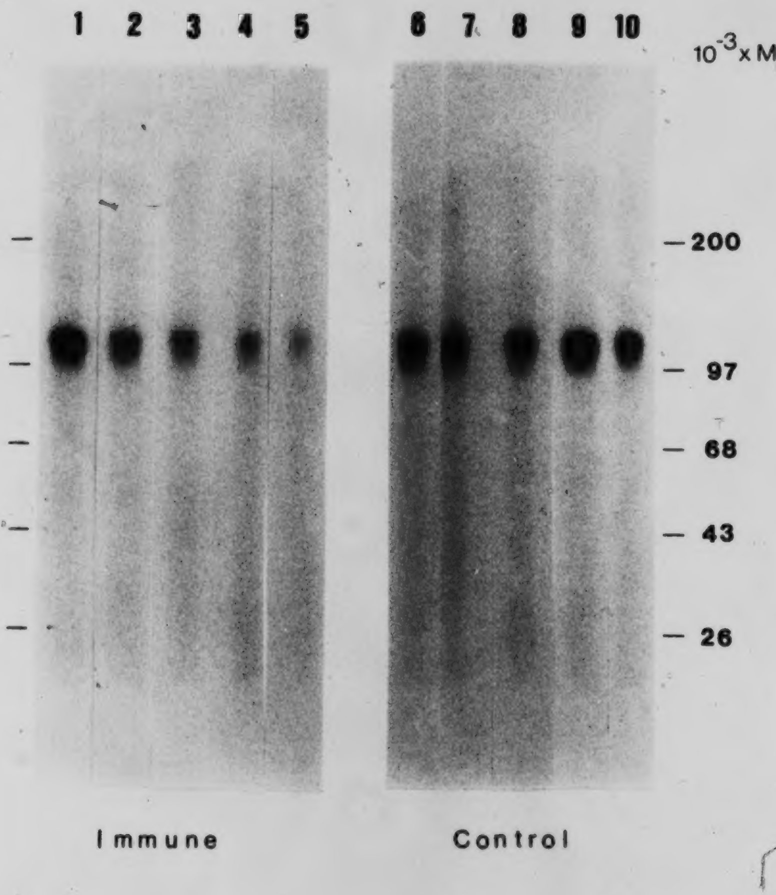

1
2
3
4
5
6
7
8
9
10
$10^{-3} \times M_r$
200
97
68
43
26
Immune
Control

FIGURE IV 6

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHICKEN LIPOVITELLIN AND PHOSVITIN

Lipovitellin was prepared from chicken egg yolks and subjected to electrophoresis on 4.5 - 18% SDS-polyacrylamide gradient gel under reducing conditions as described under "Materials and Methods". Lane A, 20 µg of lipovitellin; lane B, 10 µg of phosvitin (see "Materials"). The positions of migration of the M_r standards are indicated. Proteins were stained with Coomassie Blue containing 20 mM $AlCl_3$.

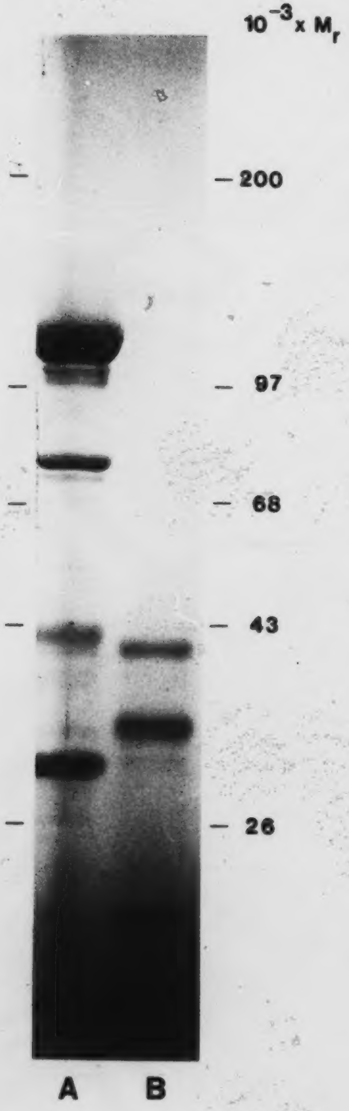

10^-3 x M_r
200
97
68
43
26
A
B

FIGURE IV 7

BINDING OF ^{125}I-CHICKEN VTG TO OOCYTE VTG RECEPTORS IS INHIBITED BY LIPOVITELLIN

Chicken oocyte membrane Triton extract (lanes 1, 3, 5, 7; 20 µg of protein/lane) and *Xenopus* oocyte membrane octylglucoside extract (lanes 2, 4, 6, 8; 100 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and ligand blotting as described under "Materials and Methods". All strips were incubated in buffer containing 2.1 µg/ml of ^{125}I-chicken VTG (250 cpm/ng) with the following additions: lanes 1 and 2, none; lanes 3 and 4, 105 µg/ml of unlabeled chicken VTG (ChVTG); lanes 5 and 6, 174 µg/ml of unlabeled chicken lipovitellin (ChLV); and lanes 7 and 8, 16 µg/ml of unlabeled chicken phosvitin (ChPV). The positions of migration of the M_r standards are indicated. Autoradiography was for 12 h.

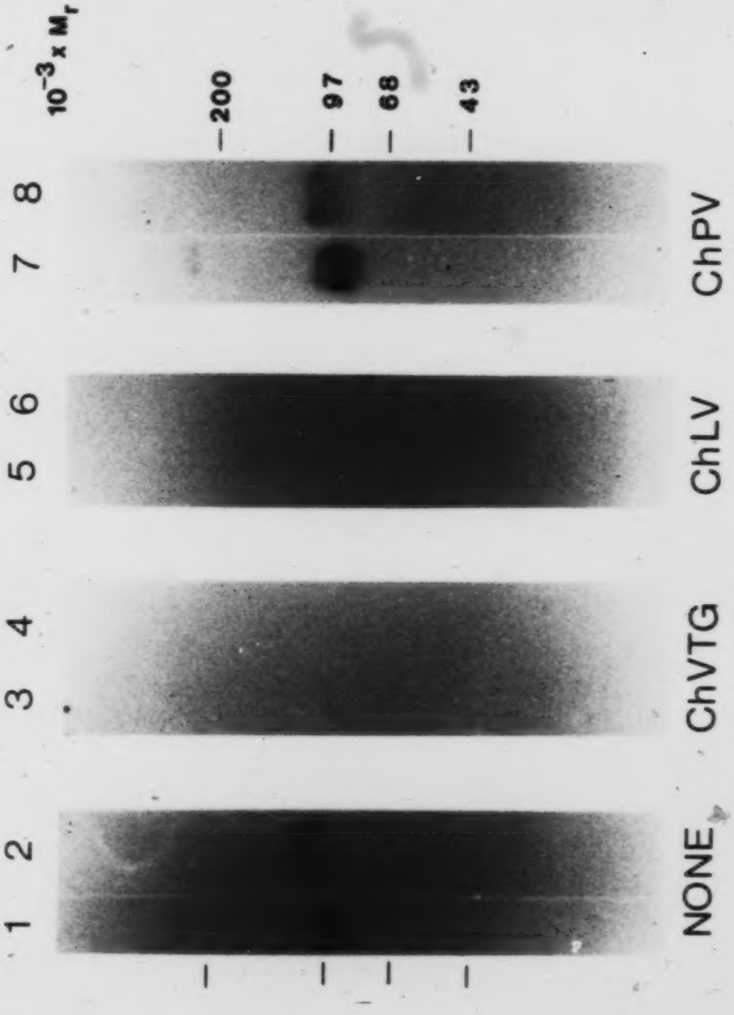

1
2
3
4
5
6
7
8
NONE
ChVTG
ChLV
ChPV
$10^{-3} \times M_r$
200
97
68
43

FIGURE IV 8

BINDING OF ^{125}I-LIPOVITELLIN TO OOCYTE VTG RECEPTORS

Chicken oocyte membrane Triton extract (lanes 1, 3, 5, 7; 20 µg of protein/lane) and *Xenopus* oocyte membrane octylglucoside extract (lanes 2, 4, 6, 8; 100 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and ligand blotting as described under "Materials and Methods". All strips were incubated in buffer containing 10 µg/ml of ^{125}I-LV (60 cpm/ng) with the following additions: lanes 1 and 2, none; lanes 2 and 3, 500 µg/ml of unlabeled chicken lipovitellin (ChLV); lanes 5 and 6, 600 µg/ml of unlabeled chicken VTG (ChVTG); and lanes 7 and 8, 88 µg/ml of unlabeled chicken phosvitin (ChPV). The positions of migration of the M_r standards are indicated. Autoradiography was for 40 h.

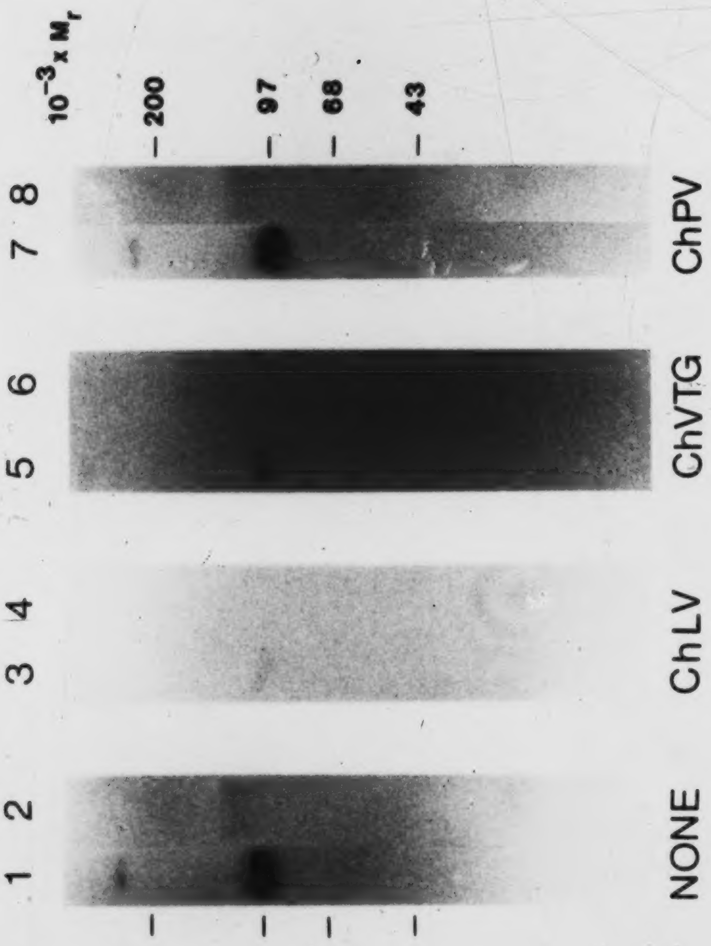
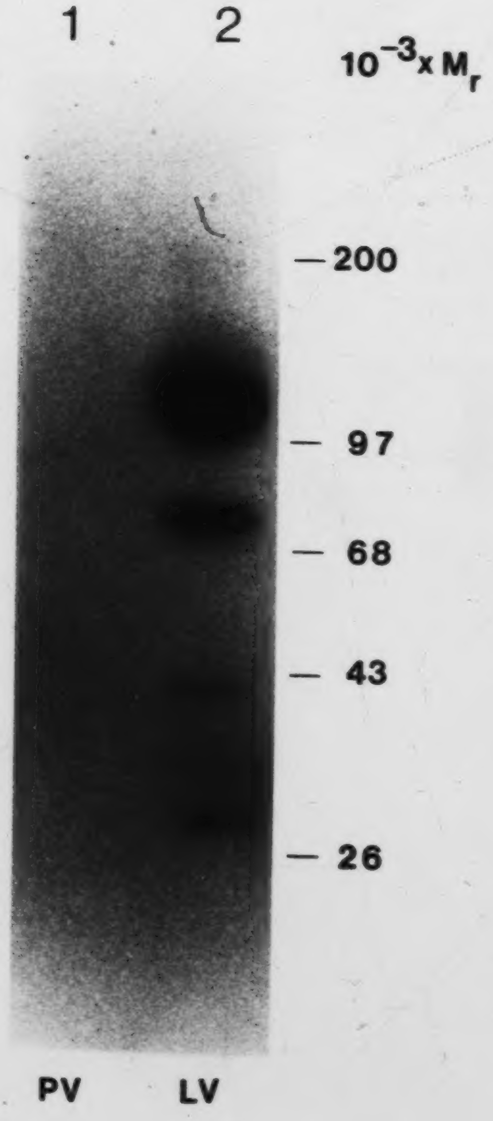

1 2
3 4
5 6
7 8
NONE
ChLV
ChVTG
ChPV
$10^{-3} \times M_r$
200
97
68
43

FIGURE IV 9

REVERSE LIGAND BLOTTING: INTERACTION BETWEEN LIPOVITELLIN POLYPEPTIDES AND CHICKEN VTG RECEPTORS

Chicken lipovitellin (lane 2; 20 µg of protein/lane) and phosvitin (lane 1; 10 µg of protein/lane) were subjected to electrophoresis under nonreducing conditions on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose as described under "Materials and Methods". The nitrocellulose was then incubated for 2 h in 20 ml of buffer containing 25 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM $CaCl_2$, 0.05% Triton X-100, 5% dry milk (Incubation Buffer). After this time, 150µl (600 µg of protein) of chicken oocyte membrane Triton extract was added and incubation was continued for 2 h at room temperature. After extensively washing with Incubation Buffer, the nitrocellulose was then incubated in the presence of 15 µg/ml of anti-OR IgG for 2 h. At the end of this incubation, the nitrocellulose was again thoroughly washed, incubated in the presence of ^{125}I-labeled protein A (1.6 µg/ml; 475 cpm/ng), and processed for autoradiography. The positions of migration of the M_r standards are indicated. Autoradiography was for 30 h.


1
2
$10^{-3} \times M_r$
200
97
68
43
26
PV
LV

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Chapter V

REGULATION OF OOGENESIS: THE PISCINE RECEPTOR FOR VITELLOGENIN[1]

INTRODUCTION

Selectivity of VTG uptake via specific oocyte receptors has recently been suggested for rainbow trout (1), but information regarding the properties of such receptors is lacking. In view of this lack of information and of the increasing interest in fish as experimental animal models (2), we decided to perform experiments aimed at characterizing oocyte VTG receptors from this source. The plasma membrane receptor for VTG was characterized from oocytes of coho salmon, *Oncorhynchus kisutch*. In direct binding studies, the receptor exhibited relatively low affinity (K_d~ 180 nM) for salmonid VTG, and by ligand blotting with radiolabeled VTG it was visualized as a protein with an apparent M_r of 100,000, under nonreducing conditions. The fish VTG receptor was shown to share key structural elements with VTG receptors from chicken and *Xenopus laevis*. Namely, cross-reactivity at the level of ligand recognition was observed among VTG receptors from these species and immunological relatedness was demonstrated by immunoblotting with anti-chicken VTG receptor antibodies. In addition, as in chicken and *Xenopus*, binding of VTG to fish oocyte receptors was shown to be mediated by the lipovitellin domain of VTG. These results clearly indicate that regulation of oocyte growth at the level of yolk formation has been accomplished by the conservation of structural features of receptors required for internalization of VTG.

[1] A version of this chapter has been published: Stifani, S., Le Menn, F., Nunez Rodriguez, J., and Schneider, W.J. (1990) *Biochim. Biophys. Acta* **1045**, 271- 273

MATERIALS AND METHODS

Materials, Animals and Diets

Sepharose 6B was from Pharmacia; all other materials were obtained from previously reported sources (3,4). White Leghorn laying hens were purchased from the Department of Animal Science, The University of Alberta, and maintained as previously described (4). Male rainbow trout, *Salmo gairdneri,* were maintained and injected with 17ß-estradiol to enhance VTG synthesis as reported (5). Vitellogenic oocytes from coho salmon (*Oncorhynchus kisutch*) were kindly provided by Dr. E.M. Donaldson, West Vancouver Laboratory, Vancouver, Canada.

Vitellogenin Purification and Radioiodination

Fish vitellogenin was obtained from the plasma of estrogen-treated male rainbow trout. Plasma was applied to a Sepharose 6B gel filtration column (150 x 1.5 cm) that had been previously equilibrated with several volumes of 100 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$, 0.5 mM phenylmethanesulfonyl fluoride (column buffer). Gel filtration was performed at 4°C at a flow rate of 16 ml/h. Those fractions shown by SDS-polyacrylamide gel electrophoresis to contain VTG were pooled and applied to a column of DEAE-cellulose. Anion-exchange chromatography was performed essentially as described for the isolation of chicken VTG (4) except that the column buffer described above was used throughout the purification steps. Elution of VTG was accomplished by means of a linear salt gradient of from 0 to 300 mM NaCl in column buffer. After elution, fractions containing VTG were pooled and stored at -70°C in aliquots after addition of a mixture of protease inhibitors (4). Chicken VTG was obtained as previously described (4). Vitellogenin preparations were radiolabeled with ^{125}I by the Iodogen method (6) to specific activities not exceeding 500 cpm/ng of protein.

Preparation of Oocyte Membranes and Solubilization of VTG Receptors

Fish oocyte membranes were prepared and solubilized in the presence of 1% Triton X-100 as described for the preparation of chicken and frog oocyte membranes (3,4).

Electrophoresis, Transfer to Nitrocellulose, and Blotting Procedures

SDS-polyacrylamide gradient slab gels were run as described (4). Electrophoretic transfer to nitrocellulose was as in (4). Ligand blotting was carried out in buffer containing 25 mM Tris-HCl (pH 7.8), 50 mM NaCl, 2 mM $CaCl_2$, and 5% non-fat dry milk unless otherwise stated. Western blotting was performed in buffer containing 25 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.05% Triton X-100, 5% non-fat dry milk; ^{125}I-protein A was used to detect bound rabbit IgG. The concentrations and specific radioactivities of the ligands and antibodies used in the incubation mixtures are indicated in the Figure Legends. Autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 films at -70°C for the times indicated in the Figure Legends.

Filter Assay for Binding of ^{125}I-VTG to Oocyte VTG Receptors

Incubation mixtures contained either precipitated oocyte membrane octylglucoside extracts (4) or crude oocyte membranes that had been resuspended in a buffer consisting of 25 mM Tris-HCl (pH 7.8), 50 mM NaCl, 2mM $CaCl_2$ to final protein concentrations of 10-15 mg/ml (see Figure Legends). Separation of free from receptor-bound ligand was achieved by filtration as previously described (4).

Other Methods

Chicken lipovitellin was prepared from the yolks of freshly laid eggs as described (3). Human high density lipoprotein (subclass 3) was obtained as described in (7).

^{125}I-protein A was obtained by the Iodogen method (6). The protein content of samples containing detergent and of vitellogenins was measured by a modification of the Lowry procedure (8). IgG fractions were purified from sera on columns of protein A-Sepharose CL-4B (4).

RESULTS

Although neither the amino acid sequence determination of fish vitellogenin nor a direct comparison of its properties with those of other vertebrate vitellogenins has been reported, amino acid composition analyses and chromatographic studies of fish VTG (for review, See ref. 9) and comparison with analogous analyses of avian and amphibian VTGs indicate structural similarities among vertebrate vitellogenins. In order to investigate if vitellogenesis in different oviparous species is achieved by means of related interactions between plasmatic yolk precursor molecules and oocyte membrane receptors, we first addressed the question of whether or not fish vitellogenins are immunologically related to their avian counterparts. To this end, Western blotting experiments were performed with a rabbit polyclonal IgG fraction that had been shown to cross-react with both chicken and *Xenopus* vitellogenins (3). Fig. V 1 shows that such antibodies not only recognized chicken VTG (lane 1), but also cross-reacted with VTG that had been purified from the plasma of the rainbow trout, *Salmo gairdneri* (lane 2). In agreement with previous studies (10), monomeric rainbow trout VTG showed an apparent M_r of 220,000 - 250,000; a smaller immunoreactive component of lower M_r was also visible and most likely represents a breakdown product of VTG. The cross-reactivity observed was very weak; this is not surprising in the light of previous reports indicating no or very distant immunological relationship not only between avian and fish VTGs but also between VTGs from different fishes (11).

We next addressed the question of whether the observed immunological relationship between chicken and fish VTGs was of functional significance for ligand-receptor interactions. For this purpose, we performed receptor-binding studies in which oocyte membranes from coho salmon and ^{125}I-labeled VTG from the closely related salmonid, the rainbow trout, were used. When binding studies were performed as described in the "Materials and Methods" section, the results shown in Fig. V 2 were obtained. Binding of radiolabeled VTG to oocyte membranes appeared to be saturable and specific (panel a); the saturable component was reduced to a nonspecific, linear component when the reaction mixtures included 5 mM suramin, a compound that had been shown to block binding of chicken VTG to chicken oocyte VTG receptors (4). Analysis of the binding data according to the method of Scatchard indicated the existence of a single binding site for VTG (panel b) with an apparent K_d of 81 μg of VTG/ml, corresponding to about 180 nM assuming a molecular weight of 450,000 for native rainbow trout VTG (10). In addition, when binding studies with ^{125}I-fish VTG were performed using membrane proteins from chicken oocytes, saturable binding was observed (Fig. V 3), suggesting that the similarity between chicken and fish vitellogenins observed in immunological studies extends to their ability to interact with VTG receptors.

To further test this hypothesis, competition binding studies were performed in which increasing concentrations of unlabeled ligands were tested for their ability to compete with radiolabeled ligands for binding to VTG receptors. Fig. V 4 shows that the binding of ^{125}I-fish VTG to chicken oocyte VTG receptors was significantly reduced when the incubation mixtures contained progressively higher amounts of unlabeled VTG from either chicken or fish. On the other hand, no competition for binding was observed when very high concentrations of human high density lipoprotein (subclass 3), an unrelated lipoprotein, were tested. Taken together, the results of these studies indicate that VTG receptors from chicken and fish oocyte membranes are in perfect rapport with each other in that both receptors recognize both ligands. Additional evidence to support this theory came from ligand blotting, a technique previously used to visualize oocyte VTG receptors from chicken and frog (3,4). Thus, membranes from both chicken and fish oocytes were prepared, membrane proteins were solubilized with Triton X-100 and fractionated by electrophoresis on a SDS-polyacrylamide gel in the absence of sulfhydryl-reducing agents, followed by electrophoretic transfer to nitrocellulose; the nitrocellulose replicas were then incubated in the presence of ^{125}I-labeled chicken VTG (Fig. V 5, Panel a). As expected, in chicken oocyte membrane extracts, a protein with an apparent M_r of 95,000 was visualized, corresponding to the previously described chicken oocyte VTG receptor (4,12) (lane 1). More significantly, ^{125}I-chicken VTG bound to a single membrane protein from fish oocytes, which migrated according to an apparent M_r of about 100,000 (Fig. V 5, lane 2). The binding of ^{125}I-chicken VTG to both membrane proteins exhibited the specificity typical for receptor-ligand interactions, since it was markedly reduced by the presence of a 50-fold excess of unlabeled vitellogenin from both chicken (lanes 3 and 4) and fish (lanes 5 and 6). Further evidence that the 100-kDa membrane protein from fish oocytes is the receptor for vitellogenin was obtained in ligand blotting experiments with ^{125}I-fish VTG as the ligand. In preliminary studies, we observed that inclusion of Triton X-100 in the incubation medium resulted in lower background binding without affecting the stability of ^{125}I-fish VTG, and thus elected to include Triton in all subsequent incubations with this ligand. Fig. V 5, Panel b shows clearly that ^{125}I-fish VTG bound exactly to the same membrane proteins as chicken VTG and that these binding reactions were specific because large excesses of unlabeled vitellogenins abolished the signals (lanes 7-12). The 100-kDa VTG-binding activity of fish oocyte membranes was totally lost when membrane proteins had been exposed to disulfide-bond reducing agents during gel electrophoresis (Stifani, S., and Schneider, W.J., unpublished observations).

The demonstration that chicken and fish VTG receptors share structural features required for vitellogenin recognition, and our previous observation that this is also true for chicken and frog oocyte VTG receptors prompted us to test whether VTG receptors from fish and amphibia are also compatible systems. Fig. V 6 shows that in ligand blotting experiments with ^{125}I-fish VTG as ligand, binding to both the 95-kDa chicken VTG receptor (lane 1) and the 115-kDa frog VTG receptor (3) (lane 2) was observed. Binding to both receptors was dramatically reduced in the presence of excess unlabeled VTG (lanes 3 and 4). Furthermore, we have previously documented that the site(s) on VTG recognized by oocyte receptors is located within the lipovitellin domain of vitellogenin (3). Here, we found that the same property is displayed by the fish VTG receptor, as shown in Fig. V 7: when ^{125}I-chicken lipovitellin was used in ligand blotting experiments, it bound to both the chicken (lane 1) and the fish (lane 2) VTG receptors. Finally, a direct immunological relationship between avian and fish VTG receptors was demonstrated by Western blotting analyses using rabbit polyclonal antibodies directed against the chicken VTG receptor (12). Fig. V 8 shows that these antibodies not only recognized their original antigen (lane 1), but also cross-reacted with the 100-kDa fish VTG receptor (lane 2).

DISCUSSION

The results presented in this chapter provide the first characterization of fish oocyte vitellogenin receptors. In particular, when membranes were prepared from oocytes of coho salmon, *Onchorhynchus kisutch*, and analyzed for their ability to bind ^{125}I-labeled VTG from the related rainbow trout, *Salmo gairdneri*, a single, saturable binding site was demonstrated (Fig. V 2). Analysis of the binding data by the method of Scatchard revealed an apparent dissociation constant of 81 μg of VTG/ml, a value similar to the K_d of 96 μg/ml reported for the binding of chicken VTG to chicken oocyte VTG receptors (4), and well below plasma VTG levels in female fish during the reproductive cycle, which have been reported to range from 5 to 80 mg/ml (13). As previously shown for other lipoprotein receptors (3,14), VTG-binding capacity was abolished by exposure to disulfide reducing agents or if the drug suramin was present in the incubation mixtures (Fig. V 2). Suramin is a polysulfated polycyclic hydrocarbon bearing a high density of negative charges and has been shown to block binding of chicken VTG (4) and locust VTG (15) to their oocyte receptors as well as of human LDL (6) and platelet derived growth factor (16) to their respective receptors. In the current studies, we have extended our observations of very similar properties of the chicken and the fish VTG receptors in that we show that these vitellogenin receptors are interchangeable and share common binding sites for their ligands (Figs. V 3 and V 4). This conservation of structural features of both ligands and receptors implicated in regulation of vitellogenesis at the level of oocyte plasma membrane internalization of circulating macromolecules is even more striking when considering the results of Fig. V 1. Namely, immunological analyses indicate that only weak cross-reactivity exists between chicken and fish VTGs as compared to the very strong cross-reactivity at the level of receptor recognition. In this context, it will be of interest to investigate if the similarity among vertebrate VTG receptors extends to invertebrate species.

By ligand blotting, the fish VTG receptor was identified as a protein with an apparent molecular weight of 100,000 after electrophoresis under nonreducing conditions; the chicken oocyte VTG receptor served as a comparative control in these experiments (Fig. V 5). Analysis of the ligand specificity of VTG receptors by ligand blotting confirmed the results of the binding studies shown in Fig. V 4. In all cases, the interaction of ligands with receptors could be effectively inhibited by the presence of a large excess of unlabeled ligand (Fig. V 5, lanes 3-6 and 9-12). Fish VTG also recognized the 115-kDa *Xenopus* oocyte VTG receptor (3) in a specific fashion (Fig. V 6), confirming the observations of these and previous studies (3) demonstrating the similarity among VTG receptors from different animals. Furthermore, we observed direct binding of radiolabeled chicken lipovitellin to the 100-kDa fish VTG receptor (Fig. V 7). Previous studies showed that isolated lipovitellin from chicken eggs is capable of binding to oocyte VTG receptors from chicken and *Xenopus,* indicating that in vertebrate animals VTG receptors show specificity for conserved sequences located on the lipovitellin portion (3).

Finally, the immunological cross-reactivity first demonstrated for avian and amphibian vitellogenin receptors (3) has been extended to fish (Fig. V 8). In the course of that work, an immunological relationship between VTG receptors from chicken and frog and mammalian low density lipoprotein receptors was demonstrated with antibodies raised against the bovine low density lipoprotein receptor; since these antibodies failed to recognize the 100-kDa protein in fish oocytes (Stifani, S., and Schneider, W.J., unpublished observations), such relationship does not appear to extend to fish VTG receptors. While the reasons for this finding remain to be elucidated, we need to consider that teleosts are more distantly related to mammals than birds or amphibia.

We conclude that regulation of vitellogenesis is a highly conserved process at the level of receptor-mediated vitellogenin uptake by growing oocytes. The pivotal role of VTG internalization in reproduction can be expected to exert considerable pressure on the evolution of ligands and receptors, resulting in gene products that share the elements necessary for mutual recognition. This development seems to have come to a breakpoint when mammals appeared. From an evolutionary standpoint, it would be interesting to determine whether in viviparous animals VTG receptors have been remodeled in order to perform new functions. In this context, the immunological relatedness of mammalian low density lipoprotein receptors and the VTG receptors from chicken and frog and the ability of the chicken oocyte VTG receptor to bind apolipoprotein B-containing lipoproteins (12) are not surprising, considering that VTG probably represents the ancestor of modern lipoproteins (17).

FIGURE V 1

IMMUNOBLOTTING OF CHICKEN AND FISH VTG

Chicken VTG (4 µg; lane 1) and rainbow trout VTG (20 µg; lane 2) were prepared and subjected to electrophoresis in the absence of reducing agents on a 4.5 - 18% SDS-polyacrylamide gradient gel as described under "Materials and Methods". After electrophoresis, proteins were transferred to nitrocellulose and the replicas were used for immunoblotting. Reaction mixtures contained 10 µg/ml of rabbit anti-chicken-VTG IgG and incubation was as described in the "Materials and Methods" section. The bound antibodies were detected by incubation with ^{125}I-labeled protein A (1.6 µg/ml; 537 cpm/ng). The positions of migration of the M_r standards are indicated. Autoradiography was for either 1 h (lane 1) or for 24 h (lane 2).

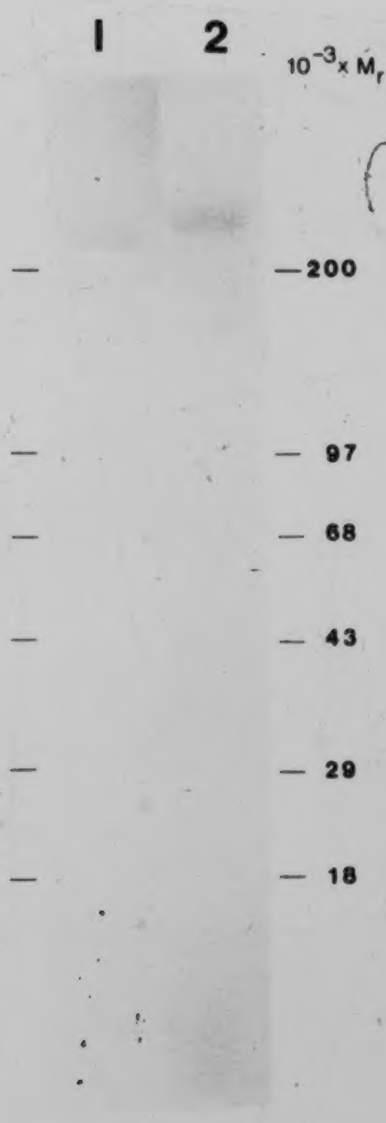

1
2
10^-3 x Mr
200
97
68
43
29
18

FIGURE V 2

BINDING OF ^{125}I-FISH VTG TO FISH OOCYTE MEMBRANES

(a) The standard assay mixture contained 165 μg of fish oocyte membrane protein in a final volume of 100 μl. Each tube contained the indicated concentrations of ^{125}I-fish VTG (43 cpm/ng) in the absence (■) or presence (▲) of 5 mM suramin. High affinity binding (□) was determined by subtraction of nonspecific binding (▲) from total binding (■). The values represent the average of duplicate incubations. (b) Scatchard plot of the high affinity binding data. The ratio bound/free is the amount of bound ^{125}I-VTG (μg of protein/ml) divided by the amount of unbound protein in the reaction mixture (μg of protein/ml).

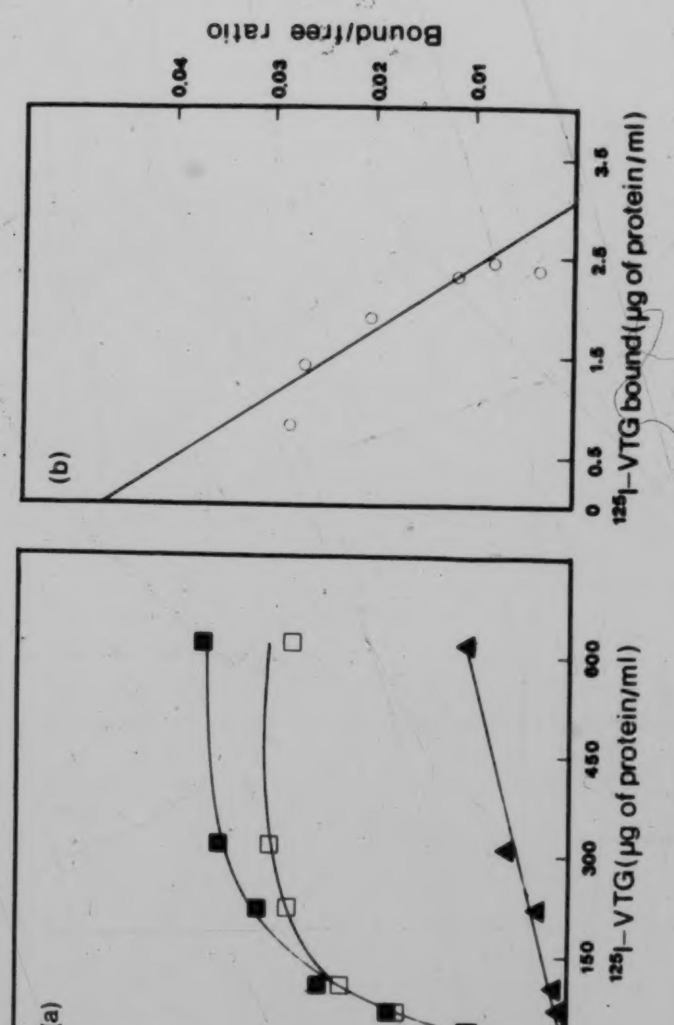

(a)
^{125}I–VTG bound(µg/mg of protein)
0
0.5
1
1.5
2
^{125}I–VTG(µg of protein/ml)
0
150
300
450
600
(b)
Bound/free ratio
0.01
0.02
0.03
0.04
^{125}I–VTG bound(µg of protein/ml)
0
0.5
1.5
2.5
3.5

FIGURE V 3

BINDING OF ^{125}I-FISH VTG TO PRECIPITATED OCTYLGLUCOSIDE EXTRACT OF CHICKEN OOCYTE MEMBRANES

Experimental conditions were as described in the legend to Fig. V 2, except that 40 µg of protein of precipitated octylglucoside extract were used instead of intact membranes. High affinity binding (▲) was determined by subtraction of nonspecific binding (△) from total binding (○).

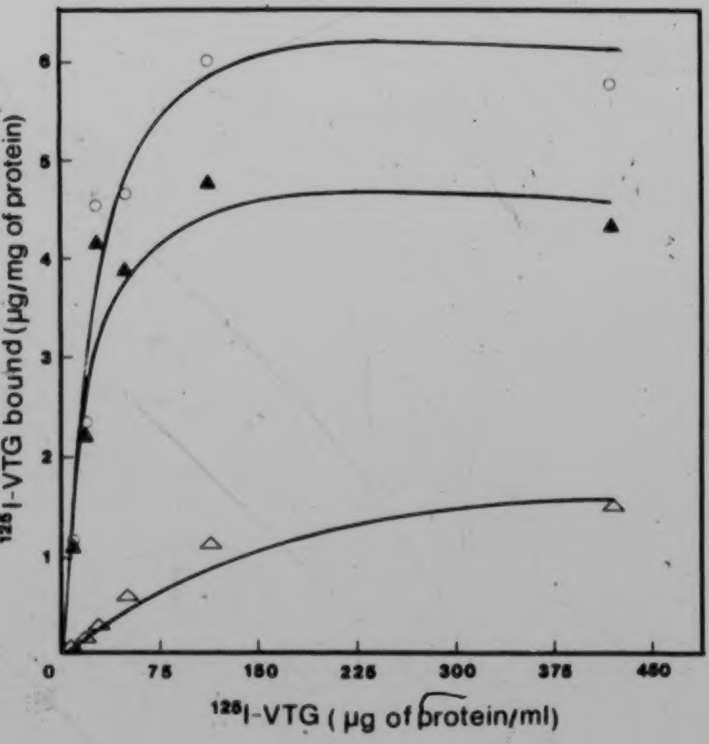

^{125}I-VTG bound (µg/mg of protein)
6
5
4
3
2
1
0
75
150
225
300
375
450
^{125}I-VTG (µg of protein/ml)

FIGURE V 4

COMPETITION OF UNLABELED LIPOPROTEINS FOR BINDING OF ^{125}I-FISH VTG TO CHICKEN OOCYTE VTG RECEPTORS

Each assay tube (final volume 150 µl) contained 30 µg of protein of precipitated octylglucoside extract, 42 µg of ^{125}I-fish VTG/ml (43 cpm/ng), and the indicated concentrations of unlabeled fish VTG (▲), unlabeled chicken VTG (△) or unlabeled human high density lipoprotein (subclass 3) (○). Specific binding was defined as described in the legend to Fig. V 2. Each point represents the average of duplicate incubations.

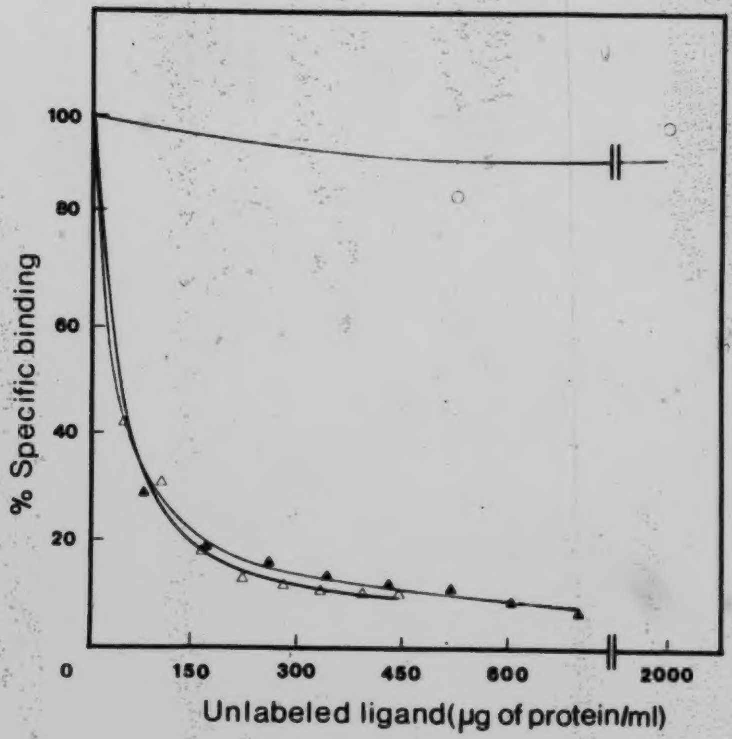

100
80
60
40
20
% Specific binding
0
150
300
450
600
2000
Unlabeled ligand(μg of protein/ml)

FIGURE V 5

LIGAND BLOTTING OF OOCYTE VTG RECEPTORS

Chicken oocyte membrane Triton extract (lanes 1, 3, 5, 7, 9, 11; 10 µg of protein/lane) and fish oocyte membrane Triton extract (lanes 2, 4, 6, 8, 10, 12; 200 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and ligand blotting as described under "Materials and Methods". In panel (a), all strips were incubated in buffer containing 6.5 µg/ml of ^{125}I-chicken VTG (463 cpm/ng) with the following additions: lanes 1, 2, none; lanes 3, 4, 325 µg/ml of unlabeled chicken VTG (ChVTG); lanes 5, 6, 325 µg/ml of unlabeled fish VTG (FVTG). Autoradiography was for 2.5 h. In panel (b), the incubation buffer contained 0.05% (v/v) of Triton X-100; all strips were incubated in buffer containing 2.0 µg/ml of ^{125}I-fish VTG (258 cpm/ng) with the following additions: lanes 7, 8, none; lanes 9, 10, 100 µg/ml of unlabeled fish VTG (FVTG); lanes 11, 12, 100 µg/ml of unlabeled chicken VTG (ChVTG). Autoradiography was for 1 h. The positions of migration of the M_r standards are indicated.

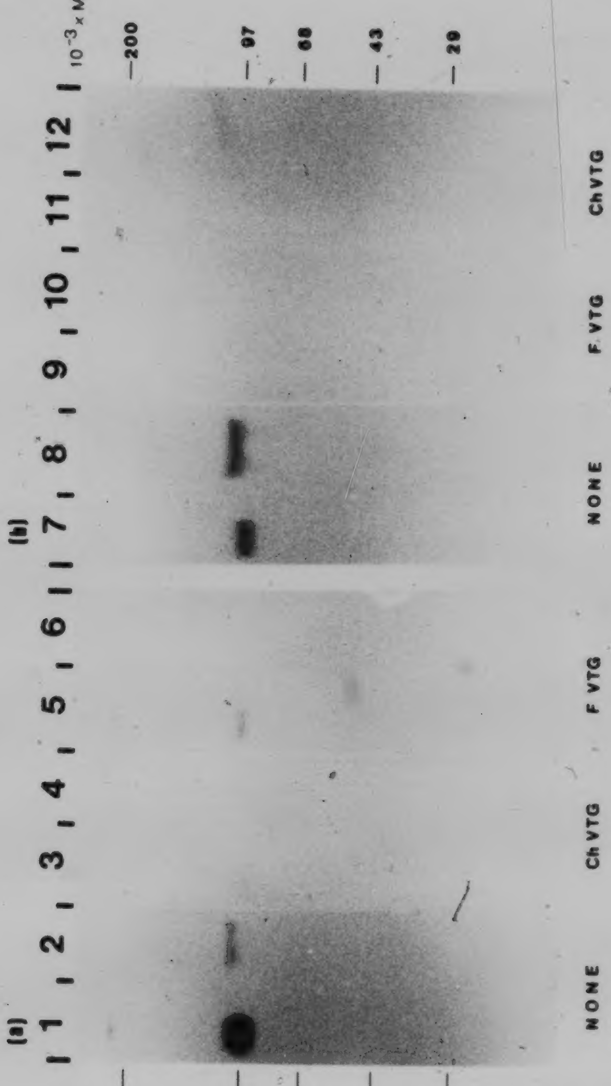

(a)
1
2
3
4
5
6
(b)
7
8
9
10
11
12
$10^{-3} \times M_r$
200
97
68
43
29
NONE
ChVTG
F VTG
NONE
F VTG
ChVTG

FIGURE V 6

BINDING OF ^{125}I-FISH VTG TO CHICKEN AND FROG VTG RECEPTORS

Chicken oocyte membrane Triton extract (lanes 1 and 3; 20 μg of protein/lane) and *Xenopus* oocyte membrane octylglucoside extract (lanes 2 and 4; 100 μg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and ligand blotting as described under "Materials and Methods". Strips were incubated in buffer containing 2.4 μg/ml of ^{125}I-fish VTG (316 cpm/ng) with the following additions: lanes 1, 2, none; lanes 3, 4, 120 μg/ml of unlabeled fish VTG (FVTG). The positions of migration of the M_r standards are indicated. Autoradiography was for 2 h.

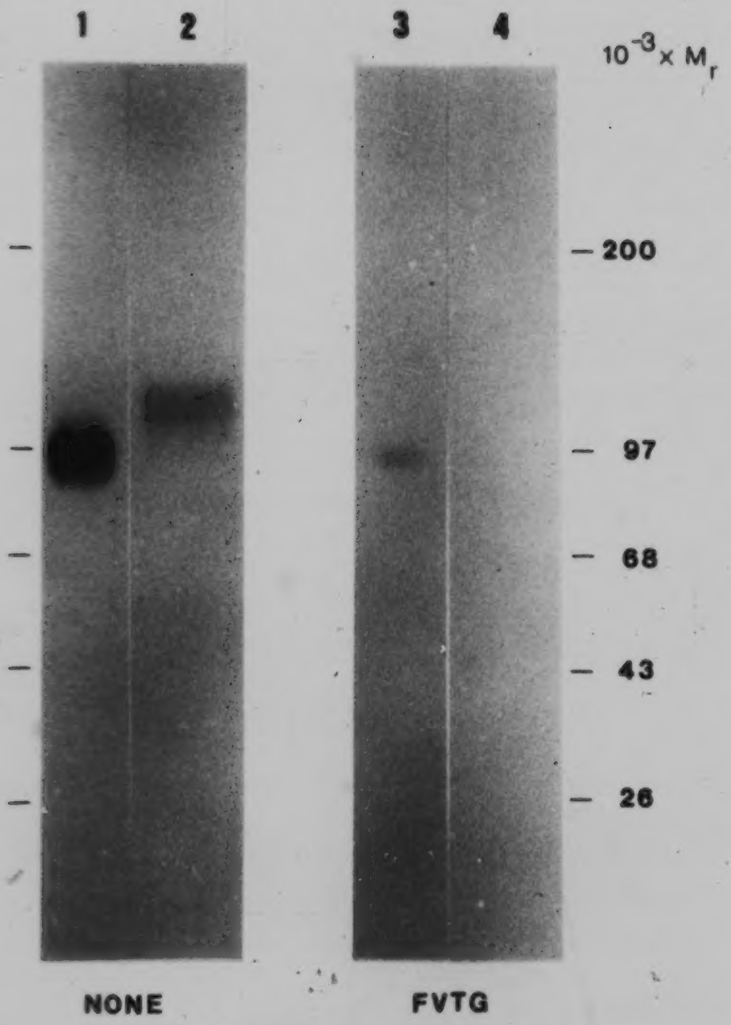

1
2
3
4
$10^{-3} \times M_r$
200
97
68
43
26
NONE
FVTG

FIGURE V 7

BINDING OF ^{125}I-LIPOVITELLIN TO OOCYTE VTG RECEPTORS

Chicken oocyte membrane Triton extract (lane 1; 10 µg of protein/lane) and fish oocyte membrane Triton extract (lane 2, 200 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose as described under "Materials and Methods". Nitrocellulose was incubated in buffer containing 25 mM Tris-HCl (pH 7.8), 100 mM NaCl, 2 mM $CaCl_2$, 5% non-fat dry milk, in the presence of 10 µg/ml of ^{125}I-chicken lipovitellin (60 cpm/ng). The positions of migration of the M_r standards are indicated. Autoradiography was for 30 h.

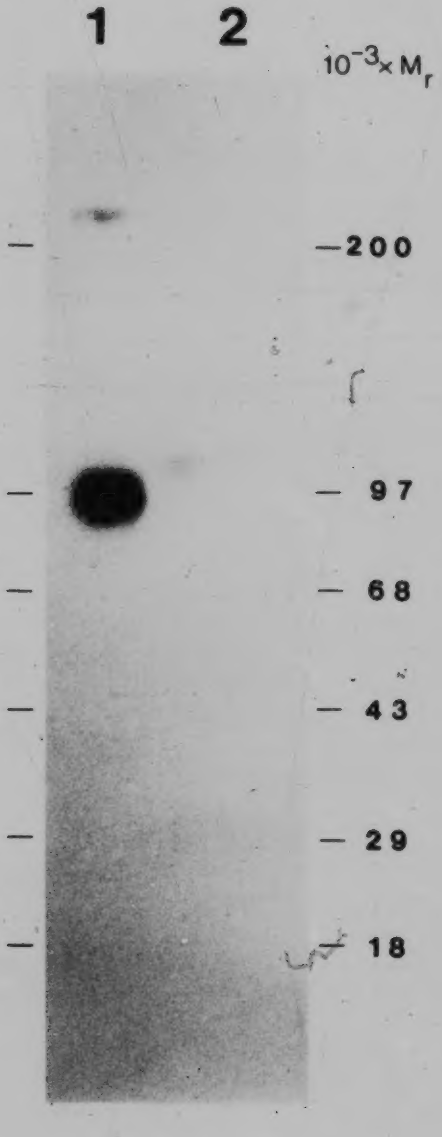
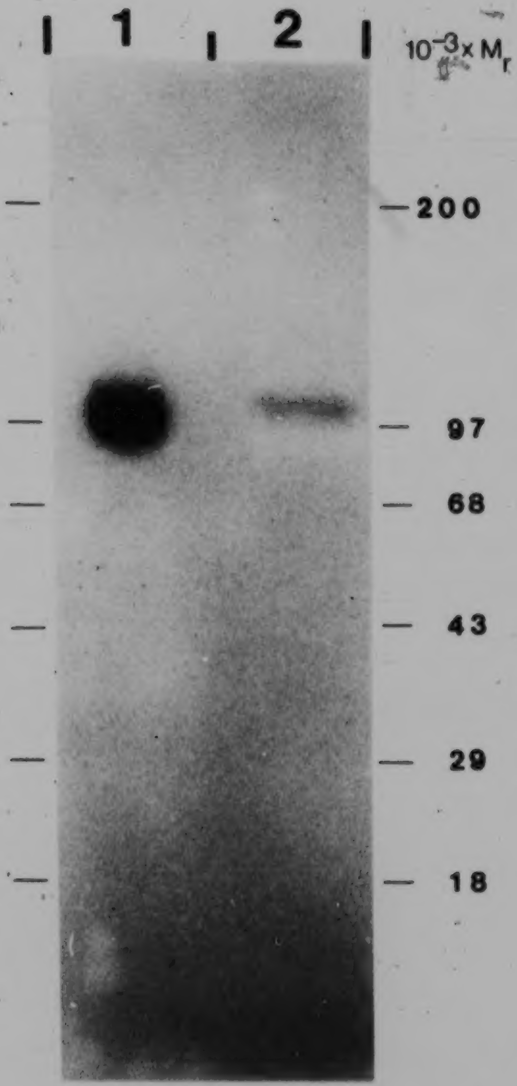

1
2
$10^{-3} \times M_r$
200
97
68
43
29
18

FIGURE V 8

IMMUNOBLOTTING OF OOCYTE VTG RECEPTORS

Chicken oocyte membrane Triton extract (lane 1; 10 µg of protein/lane) and fish oocyte membrane Triton extract (lane 2; 200 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and Western blotting as described under "Materials and Methods". Nitrocellulose was incubated in buffer containing 10 µg/ml of rabbit anti-chicken-VTG-receptor IgG, followed by ^{125}I-labeled protein A (1.6 µg/ml; 537 cpm/ng). The positions of migration of the M_r standards are indicated. Autoradiography was for 24 h.



1
2
$10^{-3} \times M_r$
200
97
68
43
29
18

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Chapter VI

THE LAYING HEN EXPRESSES TWO DIFFERENT LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEINS[1]

INTRODUCTION

Proteins belonging to the low density lipoprotein (LDL) receptor family have the ability to bind several ligands by virtue of harbouring clusters of multiple LDL receptor ligand binding repeats. At one end of the spectrum, the smallest known member of this family, and possibly the product of an ancestral gene, is the previously described chicken oocyte 95-kDa receptor (1-3). At the other end of the spectrum is the large (~600 kDa) so-called LDL receptor-related protein (LRP), which thus far has been demonstrated only in mammalian species. This protein is capable, at least *in vitro*, of interaction with the same apolipoproteins as the LDL receptor (4-6). In particular, LRP can bind certain apo-E-enriched lipoproteins (6,7), and intensive current research efforts are directed towards obtaining unambiguous proof that it functions as the long-sought-for receptor for chylomicron remnants, which are spent lipid transport vehicles of intestinal origin. Our studies of lipoprotein metabolism in the

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laying hen (1-3, 8-10) have established a powerful system to investigate the biological significance of simultaneous expression of several members of the low density lipoprotein receptor gene family within a given organism. Relevant to this aspect, we have thusfar shown that the laying hen expresses two LDL receptor family members; one of these, the 95-kDa protein, is produced only by the oocyte, while the other one (130-kDa) is restricted to somatic cells such as fibroblasts or granulosa cells (8,9). The somatic-cell receptor binds apo-B-100, has all the hallmark biochemical properties of the mammalian LDL receptor, and is part of a regulatory system for cholesterol homeostasis analogous to the LDL receptor pathway in human fibroblasts (8). The oocyte-specific receptor binds both major yolk precursors, very low density lipoprotein and vitellogenin (3), and its altered expression in a mutant non-laying strain (3,10) strongly suggests that it is a key component in yolk deposition and oocyte growth. Interestingly, this molecule is immunologically more closely related to the mammalian LDL receptor than is the chicken somatic-cell receptor (8), and, in addition, recognizes not only apo-B-100 and VTG, but also apo-E, which is not synthesized by avian species (11).

These findings, and certain common structural and functional features of apo-E and VTG have led us to hypothesize that VTG, which has evolved in oviparous species, represents a counterpart to mammalian apoE (3,11). Consequently, we feel that the oocyte receptor, an essential component of the reproductive effort of oviparous species such as the chicken, is the product of a founder gene. As such, it is endowed with structural features that allow it to interact with a range of ligands, which may have evolved similarly from a common ancestor to acquire specific modern hosts and functions. The modular structure of the LDL receptor family proteins might not only hold the key to their complex *in vitro* ligand recognition capacity, but also poses a significant challenge in delineating their true *in vivo* function(s).

Inasmuch as the avian oocyte receptor can interact with apo-B, VTG, and apo-E, could the modern mammalian LDL (apo-B/E) receptor have retained its ability to bind VTG? Do any of the other members of the LDL receptor family, and in particular LRP, show this property?

Here we show by a combination of ligand-, Ca^{2+}-, and immunoblotting that the laying hen synthesizes not one, but two different large LDL receptor-related proteins. One of these newly-found proteins has the same apparent M_r as rat liver LRP, and the other is smaller (~ 350 kDa), but is not a proteolytic product of the former. Both of these proteins, designated larger and smaller LRP, respectively, have the ability to interact with vitellogenin, a property that they share, as shown here, not only with rat LRP, but also with the LDL receptor. The larger chicken LRP was demonstrated in membranes of ovarian follicles and liver, but the smaller LRP was present only in membranes from follicles enriched for the oocyte plasma membrane. Based on these and other properties, and in analogy to the known dichotomy of chicken LDL receptors which is characterized by the production of the 95-kDa oocyte-specific receptor on one hand and the 130-kDa LDL receptor in somatic cells on the other hand (8), it appears that the smaller and larger chicken LRPs also may be restricted to the oocyte and somatic cells, respectively.

MATERIALS AND METHODS

Materials and Experimental Animals

All materials have been previously described (3) except rabbit anti-mouse IgG, which was from Cappel (West Chester, PA). White Leghorn laying hens and roosters were purchased from the Department of Animal Science, the University of Alberta, and maintained as described (1). Ovarian follicles were also collected during slaughter by permission of Lilydale Poultry Sales (Edmonton, Alberta). Male rainbow trout (*Salmo* *gairdneri*) were maintained and injected with 17ß-estradiol (12). Male rats were treated wtih 17α-ethinylestradiol as described (13).

Isolation and Radioiodination of Lipoproteins

Lipoprotein fractions (1), chicken VTG (2), and rainbow trout VTG (14) were isolated and radiolabeled with ^{125}I as described in the indicated references. Chicken VTG was reductively methylated as previously described (2).

Preparation of Membrane Fractions and Solubilization of Membrane Proteins

Ovarian follicles (3-15 mm in diameter) were surgically denuded of thecal cell layers. Membrane fractions were obtained and membrane proteins solubilized in the presence of 1% Triton X-100 as described (2). Where indicated, rat liver membrane detergent extracts were subjected to DEAE-cellulose chromatography (7) and the fractions containing the peak of LDL receptor activity, hereafter referred to as DEAE-cellulose fraction, were collected.

Electrophoresis and Blotting Procedures

One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (15) on gradient gels as indicated in the Figure Legends (2). Electrophoretic transfer to nitrocellulose, ligand blotting and immunoblotting were performed as described in (14) except that methanol was omitted from the transfer buffer, and when monoclonal antibodies were used, in which case prior to addition of ^{125}I-protein A (1.6 µg/ml; 537 cpm/ng) strips were incubated in the presence of rabbit anti-mouse IgG (2 µg/ml). Visualization of lipoprotein receptors with $^{45}CaCl_2$ was according to the procedure described in (16). The concentrations and specific radioactivities of the ligands and antibodies used in the incubation mixtures are indicated in the Figure Legends. Autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 film at -70°C for times ranging from 4 to 48 hours.

Preparation of Antibodies

Rabbit antisera directed against chicken liver LRP (see Fig. VI 6) were prepared in the following way. After fractionation of solubilized membrane proteins on a 4.5 - 18% nonreducing SDS-polyacrylamide gradient gel (using a single-lane comb) and electrophoretic transfer to nitrocellulose, the replicas were stained with Ponceau S in order to visualize the protein bands. A strip was cut out from the middle of the filters and subjected to $^{45}Ca^{2+}$ blotting in order to visualize the protein of interest. The corresponding protein band in the stained portion of the nitrocellulose replicas was localized by alignment with the autoradiograms obtained after $^{45}Ca^{2+}$ blotting. The region of the nitrocellulose replicas containing the antigen was excised, dissolved in 300 µl of dimethylsulfoxide and used for immunization of female New Zealand white rabbits as in (1). For raising antisera against the chicken follicular LRP (see Fig. VI 5), the protein was visualized by ligand blotting with ^{125}I-VTG in nitrocellulose replicas obtained from the same SDS-polyacrylamide gel, cut out from the gel after electrophoresis under nonreducing conditions, electroeluted from the gel as described (2), and used for immunization. Antisera against purified bovine LDL receptor (1) and purified chicken oocyte VTG/VLDL receptor (3) were obtained as reported previously. IgG fractions were purified from sera on columns of protein A-Sepharose CL-4B (2). The rabbit polyclonal (7) and the mouse monoclonal antibody against rat LRP used in these studies were a kind gift of Drs. R.C. Kowal, M.S. Brown, and J.L. Goldstein.

Cell Culture and Immunoprecipitation

Chicken ovarian granulosa cell layers were isolated from the largest preovulatory follicles, quickly placed into ice-cold, sterile medium A (150 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 10 mM HEPES, 0.5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4, 4 mM $NaHCO_3$, 5mM glucose, 0.0006% (W/V) phenol red (pH 7.4), and centrifuged at 4 °C at 200 x g for 5 min. After recovery, cells were washed twice (200 x g for 5 min) with medium A and then incubated in 10 ml of the same medium containing 500 µg/ml of collagenase (type IV, from Sigma). After incubation for 15 min at 37 °C with constant shaking (100 cycles/min), granulosa cells were collected by centrifugation at 200 x g for 5 min, washed twice with medium B (minimal essential medium, MEM, containing 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin), and then resuspended in 20 ml of medium B supplemented with 10% (v/v) fetal bovine serum (FBS). Cells in suspension were counted and assessed for viability by trypan blue dye exclusion. Approximately 5×10^5 cells/ml (3 ml) were seeded in 60-mm dishes and cultured at 39 °C under an atmosphere of 5% CO_2 and 95% air. Medium was changed on days 2 and 5 and experiments were initiated on day 6. Cells were washed with phosphate-buffered saline (PBS) and switched to 2 ml of methionine-free MEM containing 10% FBS, glutamine, penicillin, and streptomycin. Cells were labeled for 20 min in the presence of 84 µCi/ml of "Tran ^{35}S-label" (ICN Biomedicals; cat.no. 51006) (pulse). After this time, monolayers were either collected by scraping the dishes with a rubber policeman or further incubated for 2 hr in the presence of 2 ml of complete MEM containing 10% FBS, glutamine, penicillin, and streptomycin (chase), and then collected by scraping. Cells were lysed in 600 µl of a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM $CaCl_2$, 0.2% (w/v) SDS, 1.5% (v/v) Triton X-100, 1 mM PMSF, 2 µM leupeptin, and 2.5 µg/ml aprotinin. Lysates were centrifuged at 100,000 x g for 30 min at 4 °C and supernatants were collected. Immunoprecipitations were performed at 4 °C by incubating for 3 hr aliquots of each supernatant with 1.0 mg/ml of either anti-larger LRP IgG or control IgGs (see Fig. VI

8) in the presence of 3.5 mg/ml of bovine serum albumin. Following this time, 100 µl of a 1:1 slurry of protein A-Sepharose equilibrated in PBS containing 0.05% Triton X-100 (PBS/Triton) was added and incubation was prolonged for 2 hr. The protein A-Sepharose beads were collected by a brief centrifugation step (10 sec) in an Eppendorf microfuge and the supernatants were removed. The pellets were washed 4 times with PBS/Triton and bound material was released by addition of 90 µl of 2 x O'Farrell's buffer containing 200 mM diothiothreitol and boiling for 3 min. The samples obtained after collecting the supernatant resulting from a brief spin of the tubes were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. At the end of this step, stained gels were treated with EN^{3}HANCE (DuPont), dried, and exposed to Kodak XAR-5 films.

Other Methods

The protein content of samples containing detergent and of lipoproteins was determined as described previously (1).

RESULTS

In order to test whether the mammalian LDL receptor or other membrane proteins are capable of interaction with VTG, we performed ligand blotting with ^{125}I-labeled VTG on detergent extracts from livers of estrogen-treated rats and bovine adrenal cortex, two tissues known for significant LDL receptor activity (13, 17), and on chicken follicular membrane extracts (Fig. VI 1). As shown previously (2,3), a major VTG-binding component was identified, corresponding to 95-kDa oocyte VLDL/VTG receptor (lane 1). However, there were additional VTG-decorated bands in all of the samples: one of these, in rat (lane 2) and cow (lane 3), was of very high M$_r$

(~500,000), and a somewhat faster migrating VTG-binding protein was identified in the chicken tissue (lane 1). We had previously observed by ligand blotting on nitrocellolose membranes that VTG appeared to bind to a high-M_r protein present in chicken ovarian membrane detergent extracts (3). These results were highly variable, possibly due to difficulties in obtaining reproducible electrophoretic transfer of this large protein from the separating gel to the nitrocellulose membrane. In order to facilitate the transfer of large proteins, in the present study methanol was omitted from the transfer buffer. In both the rat and bovine samples, chicken VTG bound to an additional band with an apparent M_r of 130,000; this protein was confirmed to be the LDL receptor by ligand blotting with ^{125}I-labeled human LDL (lane 4) and its absence from control liver membranes (not shown, but cf. Figs. VI 2 and VI 4). The selective affinity for VTG of both the LDL receptor and the large protein was further demonstrated by their ability to bind not only chicken VTG, but also VTG isolated from rainbow trout plasma (lane 5); we have previously shown that there is extensive cross-species recognition of VTG by *bona fide* lipoprotein transport receptors (14, 18). Despite the large amounts of protein loaded, each membrane extract contained only two components that bound VTG, suggesting a high degree of selectivity in the observed interactions. To further test the specificity and saturability of the binding reaction, we performed the competition binding experiment shown in Fig. VI 2. The binding of ^{125}I-VTG to the two chicken proteins and to the large rat liver membrane protein (lanes 1 and 2) was competed for by unlabeled VTG (lanes 3 and 4), but not by VTG that had been reductively methylated (lanes 5 and 6), a modification that abolishes binding of LDL to its receptor (19) and the binding of VTG and VLDL to the 95-kDa oocyte receptor (1, 2). The rat liver membrane extract used in this experiment was prepared from untreated animals, and thus the LDL receptor was not visualized (cf. Fig. VI 1). However, we confirmed that the high M_r chicken follicle VTG-binding protein was smaller than that in rat liver.

In order to test the possibility that the large VTG-binding protein from chicken ovarian membranes might be related to the 95-kDa receptor by a monomer/oligomer relationship, a rabbit polyclonal antibody fraction against the high M_r ovarian VTG-binding protein visualized in Fig. VI 1 was obtained as described in "Materials and Methods". This antibody was tested for its ability to inhibit the binding of ^{125}I-VTG to oocyte membrane receptors. As shown in Fig. VI 3, lanes 1-5, after incubating nitrocellulose replicas of fractionated chicken ovarian membrane proteins with increasing concentrations of this antibody fraction, only the subsequent binding of radiolabeled VTG to the high M_r protein was inhibited, while no effect was observed on the binding of VTG to the 95-kDa receptor. In identical experiments with a nonspecific antibody preparation, no effects on binding of VTG to either protein were observed (lanes 6-10). These results suggest that the two VTG-binding proteins from chicken follicles are different proteins.

To further investigate the nature and relationship between the large VTG-binding membrane proteins in rat liver and chicken tissues, we tested whether chicken liver also expresses a high M_r protein capable of interaction with VTG. To this end, we performed ligand blotting with ^{125}I-VTG on membrane extracts of chicken follicles, livers from laying hens and roosters, and livers from untreated rats (Fig. VI 4). The data clearly demonstrate that chicken liver membranes do indeed contain a VTG-binding protein that is different from the follicle protein in that it migrates slower, *i.e.*, to the same position as the rat liver protein. Whereas chicken liver is devoid of any smaller VTG-binding protein, such as the one found in follicles, the larger protein was clearly visualized in the follicular extract upon prolonged autoradiography of lane 1 (not shown). Importantly, this novel protein is expressed in approximately equal amounts in the livers of laying hens and roosters (lanes 2 and 3). Thus, the estrogen status of the animals has no significant effect on the levels of this protein in liver, in contrast to the LDL receptor in rat (13, 20), and the endogenous hypercholesterolemia in the laying hen (in comparison to roosters) does not suppress its expression, again in contrast to the LDL receptor (7).

The regulatory features of the avian liver protein and its size, which was identical to that of the rat liver protein, suggested that the large VTG-binding proteins might represent LRP(s) (7,21). Therefore, we tested this possibility directly, and investigated the relationship between the larger chicken protein (predominantly expressed in the liver) and the smaller (exclusively follicular) protein. To this end, we performed immunoblotting with a panel of antibodies (described for reference in Table VI 1) as shown in Fig. VI 5. A monoclonal anti-rat LRP antibody decorated its antigen in rat liver membrane extract (lane 1) and two bands in chicken follicle membranes, respectively (lane 2). One of these is the chicken oocyte 95-kDa VLDL/VTG receptor, and the other comigrated with rat liver LRP. A polyclonal anti-rat LRP antibody reacted with the same two proteins in chicken follicle membranes (lane 3), but liver membranes from laying hens contained only the high-M_r antigen (lane 4), in agreement with the known exclusive expression of the 95-kDa receptor in oocytes (8-9). Rat liver LRP and the large comigrating immunoreactive chicken proteins in liver and follicle were clearly visualized, but the slightly smaller follicle protein identified by VTG-binding (Figs. VI 1, VI 2, and VI 4) showed no detectable reactivity with anti-rat LRP antibodies. However, the presence of this protein in the same follicle membrane extract was clearly shown with an antibody raised against the pure antigen (lane 6) and an antibody to the bovine LDL receptor (lane 5). As demonstrated previously (1, 2) and in Fig. VI 5, lane 5, anti-bovine LDL receptor antibodies also show crossreactivity with the 95-kDa chicken oocyte receptor. Neither of these antibodies, nor a polyclonal antibody raised against the purified 95-kDa protein recognized any of the larger VTG-binding proteins (lane 7; cf. lanes 2-4). These results (i) suggest that the large chicken proteins likely represent the avian equivalent(s) to mammalian LRP, (ii) show that the smaller chicken LRP is present in follicles, but is undetectable in liver, and

(iii) indicate that the smaller VTG-binding proteins in chicken tissues are not proteolytic products of the large protein that reacts with anti-rat LRP antibodies.

In order to obtain further biochemical evidence for the latter notion as well as for the identity of the chicken proteins with LRP, we performed the experiments described in Fig. VI 6. First, we tested whether the three proteins in the chicken follicle membrane extract would bind Ca^{2+}, a characteristic property of mammalian LRPs (4, 21). As shown in Fig. VI 6, lanes 1-5, rat LRP, both large and small chicken LRP, and the chicken oocyte 95-kDa receptor shared not only the capacity to interact with VTG, but also to bind Ca^{2+}. Again, Ca^{2+}-blotting did not reveal any major high-M_r protein in laying hen liver other than the large LRP, in agreement with the results of immuno- and VTG-blotting. Finally, we raised a rabbit polyclonal antibody to the large chicken LRP as described in "Materials and Methods", and used it to probe extracts of laying hen liver (lane 7) and follicle (lane 8) membranes for expression of crossreactive proteins (for quick reference, see Table VI 1). This IgG fraction did not cross-react with any of the other chicken membrane proteins that bind Ca^{2+} and VTG, further supporting our notion that each of the three proteins are distinct products and not derived from a large precursor. Rather, a likely proteolytic product of the large chicken LRP, different from the other visualized proteins, was identified with the anti-large chicken LRP antibody (lane 7); this fragment was not recognized by the polyclonal anti-rat LRP antibody (lane 6).

The presence in chicken ovarian membrane preparations of three different VTG-binding proteins raises the interesting question of what the individual roles of these proteins are with respect to vitellogenesis. In order to obtain some initial answers to this question, we took advantage of our observation that, in the mutant strain of chickens termed "Restricted Ovulator" (R/O), impaired binding of both VLDL and VTG to the 95-kDa receptor results in deranged oocyte growth and absence of egg laying (3,10). This finding indicated to us that the 95-kDa receptor plays a crucial role in promoting vitellogenesis and at the same time provides a way to evaluate the role of the larger VTG-binding proteins. When membrane detergent extracts from either follicles or livers obtained from laying as well as R/O hens were analyzed in immunoblotting experiments with some of the antibodies described in Table VI 1, the results shown in Fig. VI 7 were obtained. As observed previously in the case of VTG binding (3), only trace amounts of immunoreactive 95-kDa receptor could be detected in ovarian membranes from R/O hens (lane 10) when the anti-95-kDa receptor IgG was used; on the contrary, this IgG decorated a strong band on ovarian membranes from laying hens (lane 9). Also as expected, no presence of this protein could be detected in liver membranes from either source (lanes 11 and 12). Contrary to the expression pattern of the 95-kDa protein in R/O hens, no difference in the expression level of the smaller LRP was detected in ovarian membranes from laying and R/O hens using the anti-smaller LRP IgG described above (lanes 1 and 2). Similarly to the 95-kDa protein, on the other hand, the smaller LRP could not be detected in liver membrane extracts from either source (lanes 3 and 4). Finally, expression of the larger LRP was not only unaltered in ovarian membranes (lanes 5 and 6), but also in liver membranes (lanes 7 and 8) from both laying and R/O hens. Taken together, these results further support the notion that expression of the 95-kDa and smaller LRP molecules is restricted to the oocytes, while expression of the larger LRP is characteristic of different somatic tissues such as the liver. In addition, the demonstration that only the expression of the 95-kDa receptor is altered in the nonlaying R/O mutant strain supports our initial suggestion that this receptor represents a key element for completion of vitellogenesis.

We next addressed the question of whether the presence of the larger LRP in ovarian membranes could be attributed to the presence of the somatic granulosa cell layer in our membrane preparations. To this end, we prepared cultures of chicken granulosa cells (9) and performed pulse-chase studies, followed by immunoprecipitation with anti-larger LRP antibodies. Cell monolayers were pulsed for

20 min and then either directly processed for immunoprecipitation or chased for an additional period of 2 hr, after which they were collected and solubilized in the presence of detergent and the cell extracts were immunoprecipitated. As shown in Fig. VI 8, immediately after completion of the pulse period, the anti-larger LRP IgG precipitated a high-M_r protein (lane 1) that was converted to a faster migrating form after the chase period (lane 2). This situation is identical to the one observed for human LRP: this protein is synthesized as a precursor that migrates with an apparent molecular weight of 600,000 and is then proteolytically cleaved to generate two mature subunits with apparent molecular weights of 515,000 and 85,000, respectively (22). In our experiments, we could only detect the larger LRP subunit because our anti-chicken LRP antibody had been obtained after immunization of rabbits with the 515-kDa form separated after gel electrophoresis of chicken liver membrane proteins. In control experiments in which either the anti-smaller LRP IgG (lanes 3 and 4) or an antibody obtained from nonimmunized rabbits (lanes 5 and 6) were used, no radioactive polypeptides were specifically immunoprecipitated which exhibited electrophoretic mobility compatible with any of the VTG-binding proteins described above. The additional strong band observed in all our immunoprecipitates at a position corresponding to an apparent M_r of 220,000 represents the product of an unrelated gene expressed in granulosa cells which interacts equally well with each antibody fraction tested. It most likely corresponds to a secreted protein, since its cellular amount was significantly reduced after the chase period (lanes 2, 4, and 6), concomitant with its appearance in the extracellular medium (Stifani, S., and Schneider, W.J., unpublished observations). In summary, these results show that the anti-chicken larger LRP antibody can specifically precipitate from cultured ovarian granulosa cells a protein that undergoes the kind of posttranslational processing described for human LRP (22), thus indicating that these cells are a site of synthesis of the larger LRP.

DISCUSSION

In the current studies, we have made several novel observations in regards to members of the LDL receptor family. First, based on results from immunological and biochemical experiments we conclude that the chicken expresses LRP, a protein thus far believed to be restricted to mammalian species. This is of particular interest since this large protein has been implicated, among other possibilities, as the receptor for chylomicron remnants, which are lipoprotein particles not produced in avian species. Second, the laying hen synthesizes two different LRPs which are immunologically unrelated but have several characteristic properties in common. One of these proteins has the same apparent M_r as rat liver LRP, and is expressed predominantly in liver; the other protein is smaller, and is expressed in ovarian follicles, but not in the liver. This finding raises the exciting possibility that in analogy to the known somatic-cell/oocyte LDL receptor dichotomy in the laying hen (8), synthesis of the two different LRPs may also be restricted to somatic cells and the oocyte, respectively. Third, the data support the general concept that the modular structure of LDL receptor family proteins allows them to interact with multiple ligands; vitellogenin, previously shown to bind to the LDL receptor-related chicken oocyte 95-kDa lipoprotein receptor, is also recognized by mammalian LDL receptors and, possibly more importantly, by chicken and mammalian LRPs. In the following, these aspects will be discussed in more detail.

We have previously shown that the chicken oocyte 95-kDa lipoprotein receptor not only binds its physiological ligands VLDL and VTG (3), but also apo-E, a protein not synthesized by chickens (11). Despite its obvious lack of physiological relevance, the latter finding is of significance when considering evolutionary aspects of lipoprotein receptors and their ligands, and raises several interesting questions. As discussed previously (11), apo-E and VTG share certain structural and functional features that suggest that VTG, which has evolved in oviparous species, represents a counterpart to mammalian apo-E. Besides a strong cross-taxa relationship between these lipoprotein ligands, there is a direct relationship between the chicken oocyte receptor and the mammalian LDL receptor family, as evidenced by a characteristic set of common biochemical properties (1, 8) and immunological cross-reactivity (1,2,8). The present results extend these observations, in that they show that VTG can also bind to products of the mammalian LDL receptor gene family, namely the LDL receptor and LRP. It is tempting to speculate that the oocyte receptor, a key component in the reproductive mechanism of oviparous species (which evolved before mammals), is the product of a founder gene which combines elements found in different modern lipoprotein receptors that function in somatic cells. Interestingly, this receptor is the smallest competent member of the family, compatible with younger relatives having arisen by gene duplication events. The modular structure of the LDL receptor gene and protein, also compatible with this notion, holds a likely explanation for the highly conserved *in vitro* binding properties of the members of this gene family; the levels of expression , tissue- and subcellular distribution, and accessibility to the ligands might determine each molecule's function *in vivo.*

While these findings shed new light on evolutionary aspects of lipoprotein metabolism in general, the revelation that the laying hen synthesizes two different LRPs is of significant interest with regard to metabolism and development of oviparous species in particular. Extensive immunological and biochemical evidence is presented here that two high-M_r proteins, initially identified by their ability to bind VTG, are the chicken counterparts to mammalian LRP. First, as expected from being related to the smaller members of the LDL receptor family, in particular the oocyte VLDL/VTG receptor, these proteins bind not only VTG, but also apo-B-100 and apo-E (not shown, but cf. Refs. 3, 11); second, they share with the LDL receptor and rat LRP the property of binding Ca^{2+}; third, monoclonal and polyclonal antibodies directed against either rat LRP or bovine LDL receptor recognize these proteins. The most surprising finding, however, is the expression of two different LRP molecules in the chicken. While the larger one comigrated with rat LRP heavy chain (515-kDa on SDS polyacrylamide gels, ref. 22), the second LRP migrated as a ~350-kDa protein. The tissue distribution of the two forms was of particular interest to us: the smaller LRP was present in large amounts in membranes prepared from follicles which had been surgically denuded of most, but not all, follicular cells. This membrane fraction consisted of the plasma membrane of the oocyte and membranes derived from the most tightly adherent granulosa cell monolayer and some thecal fibroblasts; *i.e.*, it was enriched for zygotic plasma membrane relative to somatic cell membranes. The larger LRP was present in easily detectable amounts in liver membrane extracts, and also in extracts of follicle membranes, albeit in lower amounts. Furthermore, the smaller LRP was not detected in membranes derived from somatic cells, such as the liver (Figs. VI 4-VI 7) or cultured ovarian granulosa cells (Fig. VI 8), while the larger LRP could be immunoprecipitated from cell extracts of cultured granulosa cells after metabolic labeling (Fig. VI 8). This tissue distribution is strikingly reminiscent of that of another pair of related, but different proteins in the laying hen, namely the 95-kDa receptor for VLDL and VTG in the oocyte (1-3) on one hand, and the somatic cell-specific 130-kDa receptor on the other hand (8,9). Similarly, we believe that the smaller LRP is a zygotic product, whereas the larger LRP is produced by somatic cells. Direct evidence against the smaller LRP being a tissue-specific proteolytic product of the larger form is provided by the absence of mutual immunological cross-reactivity (Figs. VI 5 and VI 6).

Based on these and our previous findings of a somatic cell/zygote dichotomy at the chicken LDL receptor locus (8), the possibility exists that there are altogether four related gene products in the laying hen: one pair consisting of the 130-kDa somatic apo-B specific receptor (8,9) and the 95-kDa oocyte receptor for VLDL/VTG (1-3), and the other pair being the two LRPs described herein. Whether the ability of these two novel proteins to bind VTG points to their physiological function or merely reflects their kinship to the LDL receptor family will be the focus of future studies. These investigations will have to take into account the following additional observations. First, it is surprising not only that mammalian LRP recognizes a nonmammalian lipoprotein, but also that chickens, which lack apo-E, express similar proteins. This suggests that, if LRP is indeed functional in lipoprotein transport, its involvement extends beyond chylomicron remnants metabolism. Namely, the capacity of LRPs to bind a variety of protein-lipid complexes and their known tissue distribution and complex itinerary (4,6,7,21) may be reconciled with a role of LRP in intra- or intercellular, rather than or in additon to systemic lipid transport. Second, the very recent discovery of the identity of human LRP with the human α_2-macroglobulin receptor (23 and Chapter VIII) suggests that LRPs may be able to perform multiple functions, including and extending beyond the transport of lipoprotein particles. Third, since the 95-kDa receptor appears to be crucial for efficient vitellogenesis, the significance of a second oocyte protein with similar binding properties must be elucidated. Conceivably, if both proteins were indeed transport-competent *in vivo*, they may complement each other temporally, topologically, or through carrying different ligands. Alternatively, they may cooperate, especially during the extremely rapid last growth phase of the oocyte. Finally, these investigations will have to address the function of all these simultaneously expressed lipoprotein receptors in the chicken. A testable hypothesis is that the large chicken LRP and the 130-kDa somatic cell receptor act in analogy to mammalian LRP and LDL receptor, respectively, whereas in the laying hen the smaller LRP and the 95-kDa oocyte receptor in concert divert the flow of a plethora of ligands to the growing oocytes.

FIGURE VI 1

VISUALIZATION OF RAT, BOVINE, AND CHICKEN LRPs BY LIGAND BLOTTING

Chicken follicle membrane extract (lane 1; 20 μg of protein/lane), DEAE-cellulose fraction of estrogen-treated rat liver (lanes 2, 4, and 5; 400 μg of protein/lane), and bovine adrenal cortex membrane extract (lane 3; 300 μg of protein/lane) were subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel under nonreducing conditions, followed by transfer to nitrocellulose and ligand blotting. Strips in lanes 1-4 were incubated with ^{125}I-chicken VTG (22 μg/ml; 46 cpm/ng). After autoradiography, the nitrocellulose strip in lane 4 was incubated with ^{125}I-labeled human LDL (5 μg/ml; 69 cpm/ng). In lane 5, a nitrocellulose replica from a different gel was incubated with ^{125}I-VTG from rainbow trout (^{125}I-RT VTG; 5 μg/ml; 258 cpm/ng). ● , ▲ -LRP's; ○ -chicken oocyte VTG/VLDL receptor; △ -LDL receptor. The position of migration of molecular size standards are indicated.

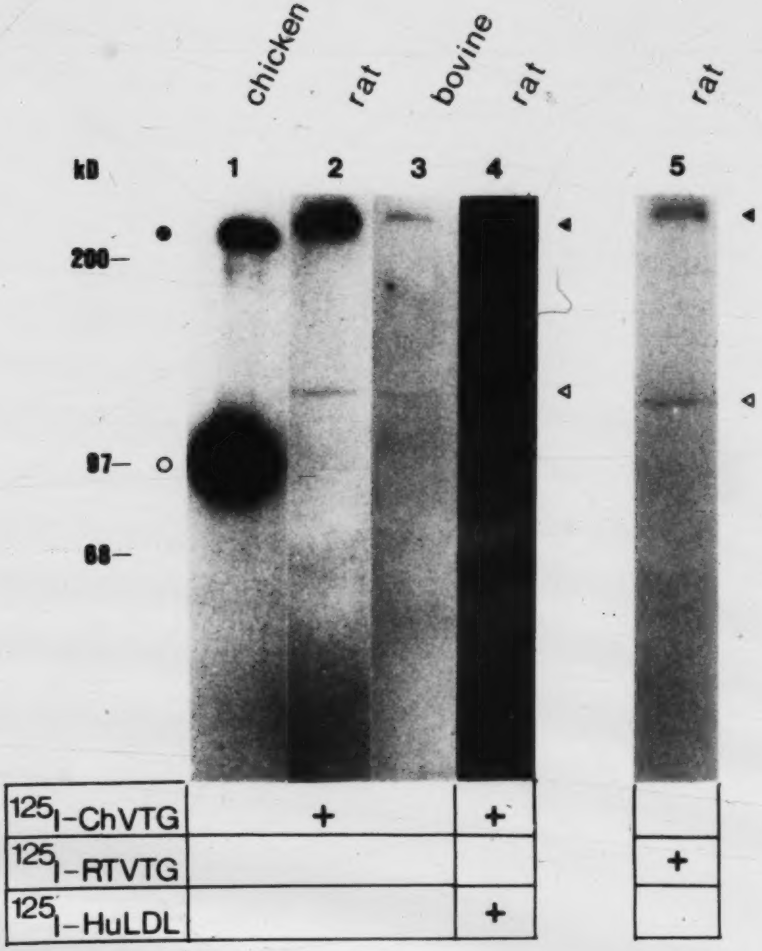
chicken
rat
bovine
rat
rat
kD
1
2
3
4
5
200—
97—
68—
^{125}I-ChVTG
+
+
^{125}I-RTVTG
+
^{125}I-HuLDL
+

FIGURE VI 2

SPECIFICITY OF VTG BINDING

Chicken follicle membrane Triton extract (lanes 1, 3 and 5; 20 μg of protein/lane) and DEAE-cellulose fraction of untreated rat liver (lanes 2, 4 and 6; 250 μg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose and ligand blotting. All lanes were incubated with ^{125}I-VTG (5 μg/ml; 46 cpm/ng) with the following additions: lanes 1 and 2, none; lanes 3 and 4, 300 μg/ml of unlabeled VTG; and lanes 5 and 6, 300 μg/ml of unlabeled reductively methylated VTG (meVTG). Molecular size standards are indicated.

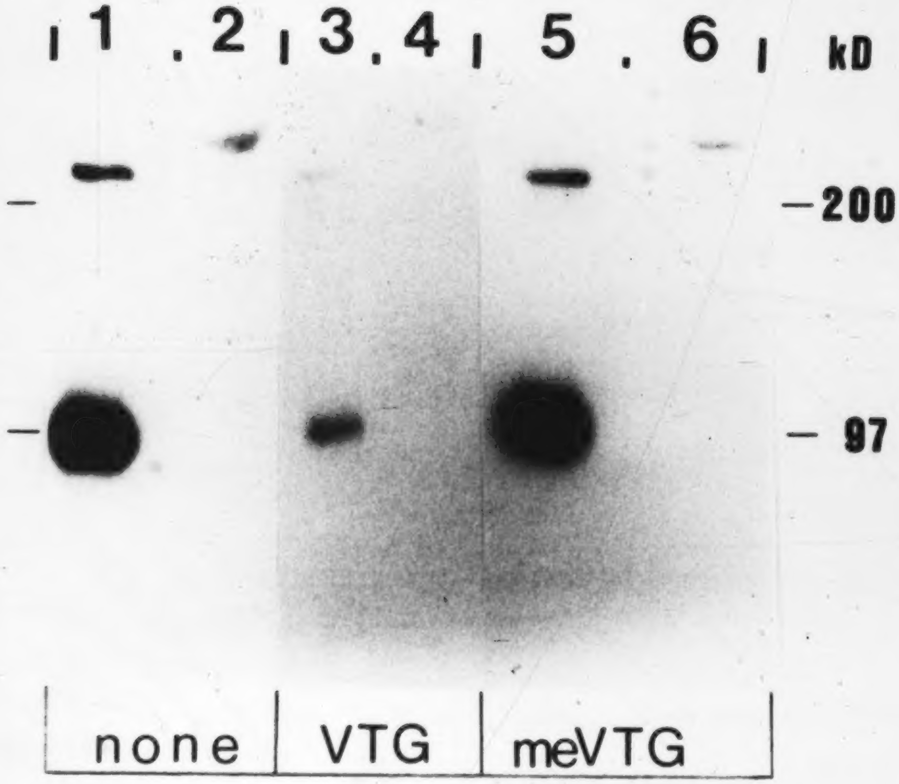
1
2
3
4
5
6
kD
200
97
none
VTG
meVTG

FIGURE VI 3

SELECTIVE INHIBITION OF ^{125}I-VTG BINDING TO HIGH MOLECULAR WEIGHT FOLLICULAR VTG-BINDING PROTEINS BY A SPECIFIC ANTIBODY

Chicken follicular membrane Triton extract (30 µg of protein/lane) was subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel and transfer to nitrocellulose. Nitrocellulose strips were incubated in ligand blotting buffer in the presence of either anti-smaller LRP IgG (lanes 2-5) or control IgG (lanes 7-10) at the following concentrations: lanes 2 and 7, 100 µg/ml; lanes 3 and 8, 200 µg/ml; lanes 4 and 9, 400 µg/ml; lanes 5 and 10, 800 µg/ml. Lanes 1 and 6 received no antibodies. At the end of the incubation, all strips were extensively washed and then incubated in the presence of 6.2 µg/ml of ^{125}I-VTG (46 cpm/ng).

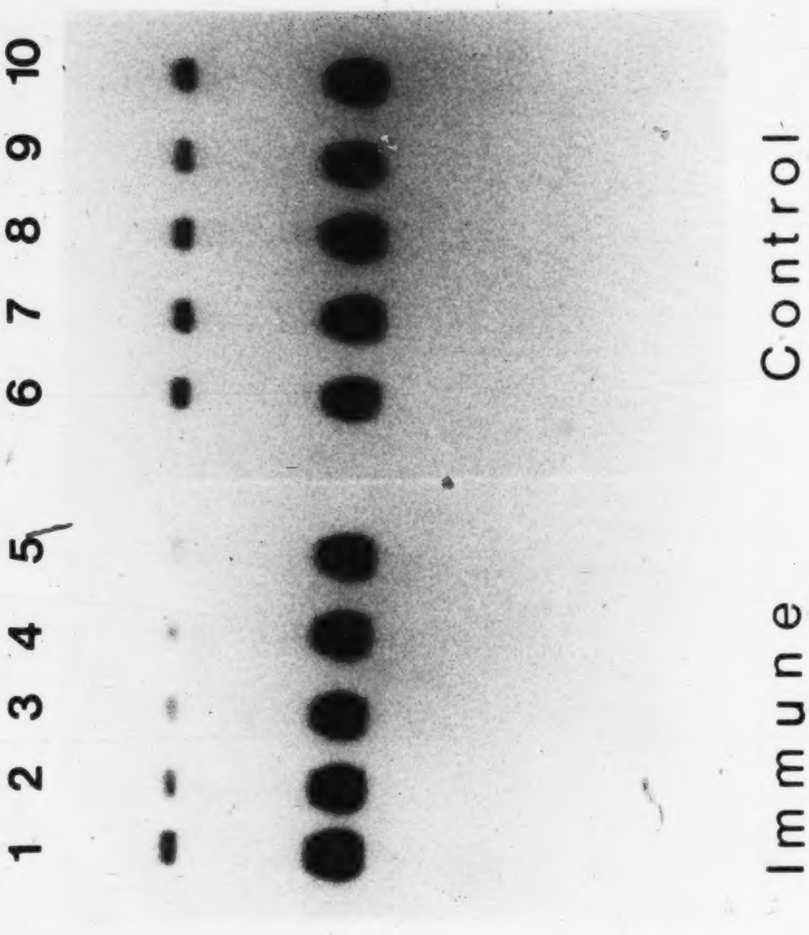
1
2
3
4
5
6
7
8
9
10
Immune
Control

FIGURE VI 4

BINDING OF VTG TO HEPATIC LRPs

Chicken follicle membrane Triton extract (lane 1, 20 µg of protein/lane), laying hen liver membrane Triton extract (lane 2; 450 µg of protein/lane), rooster liver membrane Triton extract (lane 3; 450 µg of protein/lane), and DEAE-cellulose fraction of untreated rat liver (lane 4; 250 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS polyacrylamide gradient gel, followed by transfer to nitrocellulose and ligand blotting in the presence of 10 µg/ml of ^{125}I-VTG (61 cpm/ng). F., follicle.

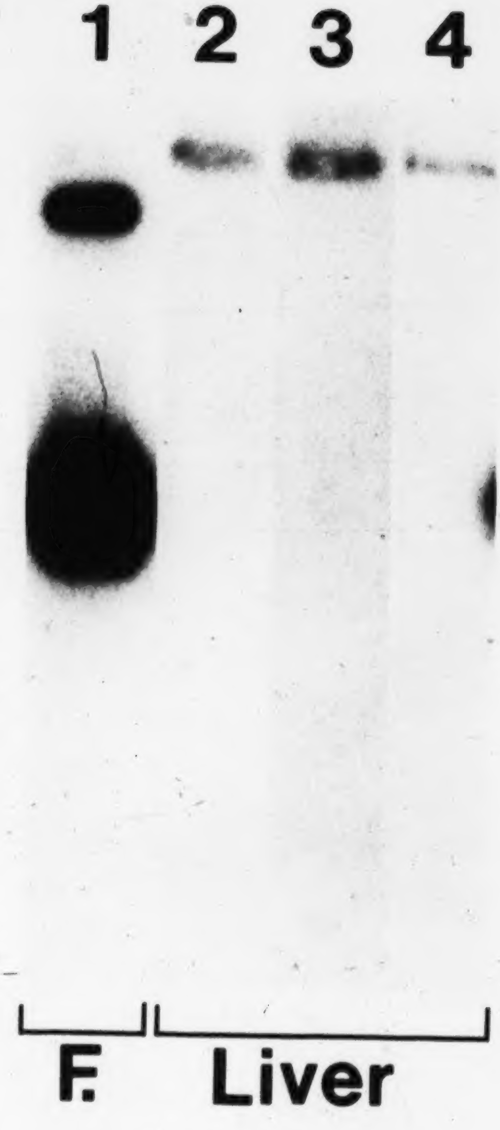
1
2
3
4
F.
Liver

FIGURE VI 5

IMMUNOBLOTTING OF CHICKEN LRPs

Membrane Triton extracts from liver of untreated rats (lane 1; 400 µg of protein/lane), chicken follicles (lanes 2, 3, 5, 6 and 7; 200 µg of protein/lane), and laying hen liver (lane 4; 400 µg of protein/lane) were subjected to electrophoresis on a 4.5 - 18% SDS-polyacrylamide gradient gel, followed by transfer to nitrocellulose and immunoblotting. Incubation was with either a monoclonal antibody directed against LRP (lanes 1 and 2; 7 µg of IgG/ml) or the following polyclonal antibodies: anti-rat LRP (lanes 3 and 4: 5 µg of IgG/ml) anti-bovine LDL receptor (lane 5; 15 µg of IgG/ml), anti-chicken smaller LRP (lane 6; 15 µg of IgG/ml), and anti-chicken VTG/VLDL receptor (lane 7; 15 µg of IgG/ml). Symbols shown are as described in the legend to Fig. VI 1.

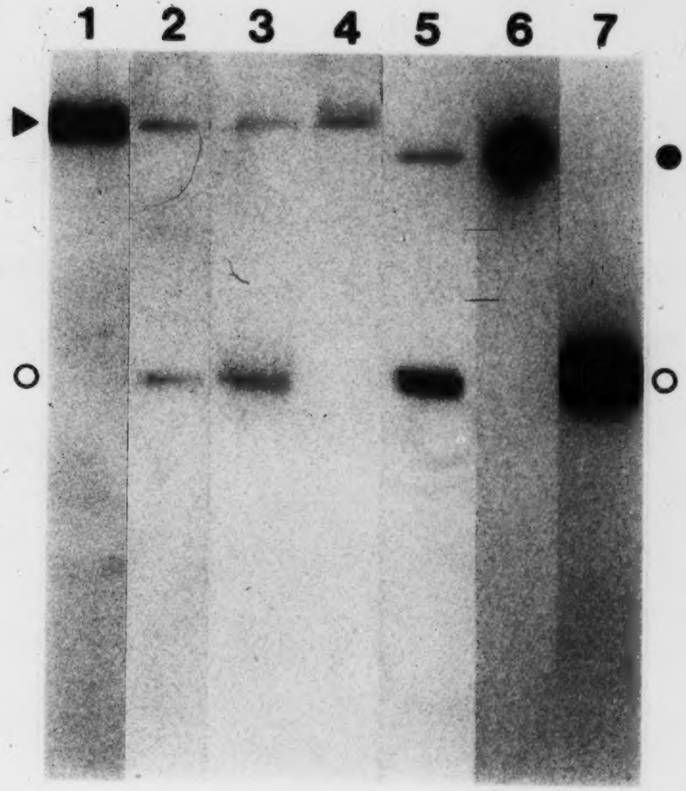
1
2
3
4
5
6
7
▶
●
○
○

FIGURE VI 6

LIGAND-, Ca^{2+}- AND IMMUNOBLOTTING OF RAT AND CHICKEN LRPs

Chicken follicle membrane Triton extract (lanes 1,4, and 8; 200 µg of protein/lane), chicken liver membrane Triton extract (lanes 2 and 5, 400 µg of protein/lane; and lanes 6 and 7, 200 µg of protein/lane) and untreated rat liver membrane Triton extract (lane 3; 400 µg of protein/lane) were subjected to electrophoresis on a 3 - 8% SDS-polyacrylamide gradient gel followed by transfer to nitrocellulose. Lanes 6 and 7 were obtained by cutting into half the nitrocellulose replica of a single electrophoretic lane. Ligand blotting (lanes 1-3) with 2.4 µg/ml of ^{125}I-VTG (387 cpm/ng), $^{45}CaCl_2$-blotting (lanes 4 and 5; 1 µCi/ml), and immunoblotting with either a polyclonal antibody against rat LRP (lane 6; 5 µg of IgG/ml), or a polyclonal antibody against the larger chicken LRP (lanes 7 and 8; 15 µg of IgG/ml) were all performed as described under "Materials and Methods". Symbols shown are as described in Fig. VI 1.

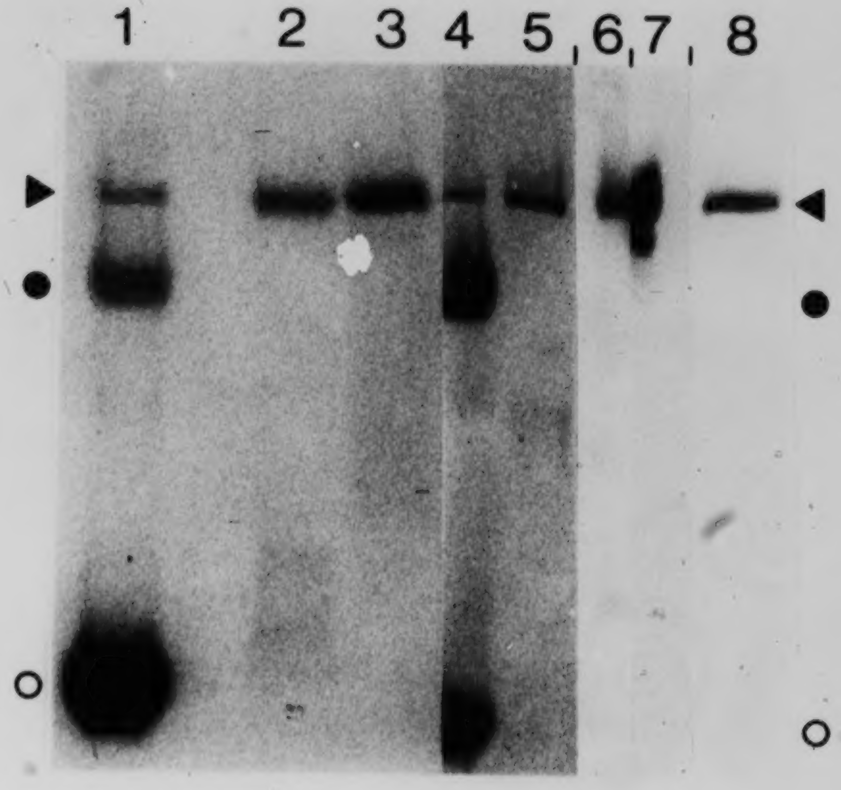
1
2
3
4
5
6
7
8
▶
●
○
◀
●
○

FIGURE VI 7

IMMUNOBLOTTING OF VTG-BINDING PROTEINS FROM LAYING AND R/O HENS

Follicular membrane Triton extract from either laying hens (lanes 1, 5, and 9; 450 µg of protein/lane) or R/O hens (lanes 2, 6, and 10; 450 µg of protein/lane) and liver membrane Triton extract from either laying hens (lanes 3, 7, and 11; 350 µg of protein/lane) or R/O hens (lanes 4, 8, and 12; 350 µg of protein/lane) were subjected to electrophoresis on a 3-12% SDS-polyacrylamide gradient gel and transfer to nitrocellulose. Immunoblotting was in the presence of 10 µg/ml of either anti-smaller LRP IgG (lanes 1-4), anti-larger LRP IgG (lanes 5-8), or anti-95-kDa receptor IgG (lanes 9-12). Bound IgG was visualized by incubation with ^{125}I-protein A. Symbols used are as described in Fig. VI 1.

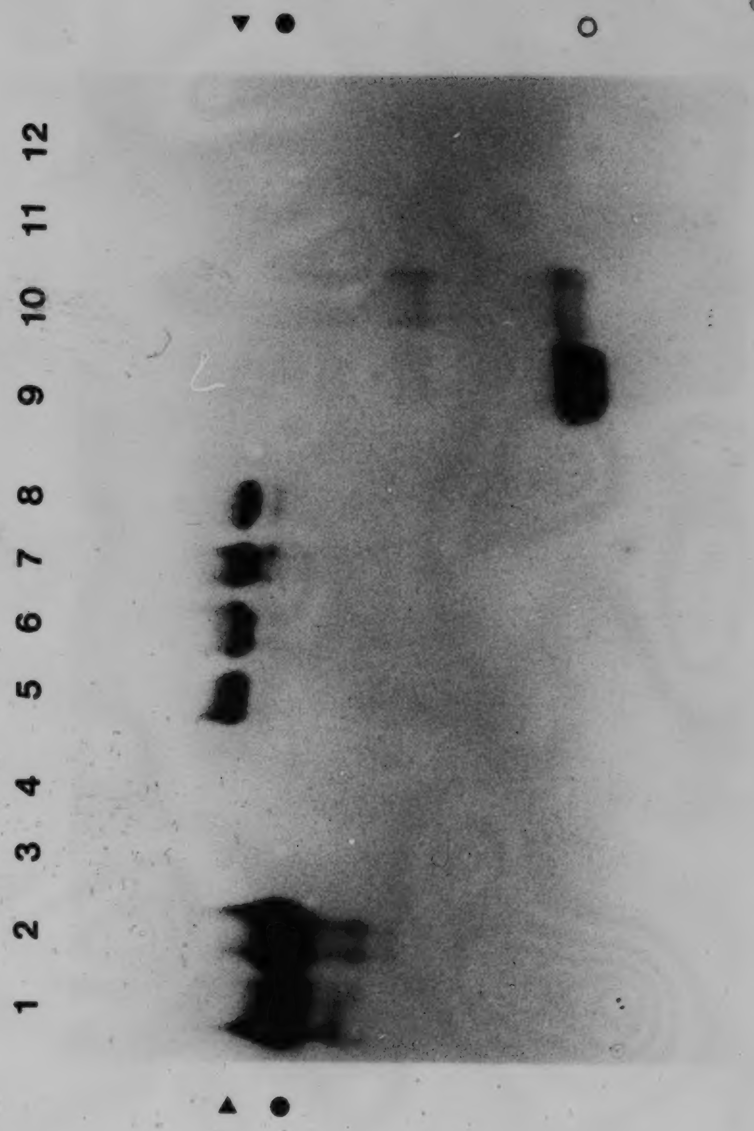
1
2
3
4
5
6
7
8
9
10
11
12
▼ ●
○
▲ ●

FIGURE VI 8

IMMUNOPRECIPITATION OF METABOLICALLY LABELED LARGER LRP FROM CHICKEN GRANULOSA CELLS

Chicken ovarian granulosa cell monolayers were obtained and cultured as described in "Materials and Methods". Cells were pulse-labeled for 20 min in the presence of 84 μCi/ml of "Tran^{35}S-label", then chased for 2 hr, after which detergent-solubilized cell extracts were immunoprecipitated with either anti-larger LRP IgG (lanes 1 and 2), anti-smaller LRP IgG (lanes 3 and 4), or nonimmune IgG (lanes 5 and 6). The immunoprecipitates were collected by incubation with protein A-Sepharose and centrifugation, and samples were subjected to SDS-polyacrylamide gel electrophoresis on a 3-12% gradient gel as described in "Materials and Methods". The stained gel was treated with EN^{3}HANCE and exposed to Kodak XAR-5 film. The position of migration of molecular size standards are indicated.

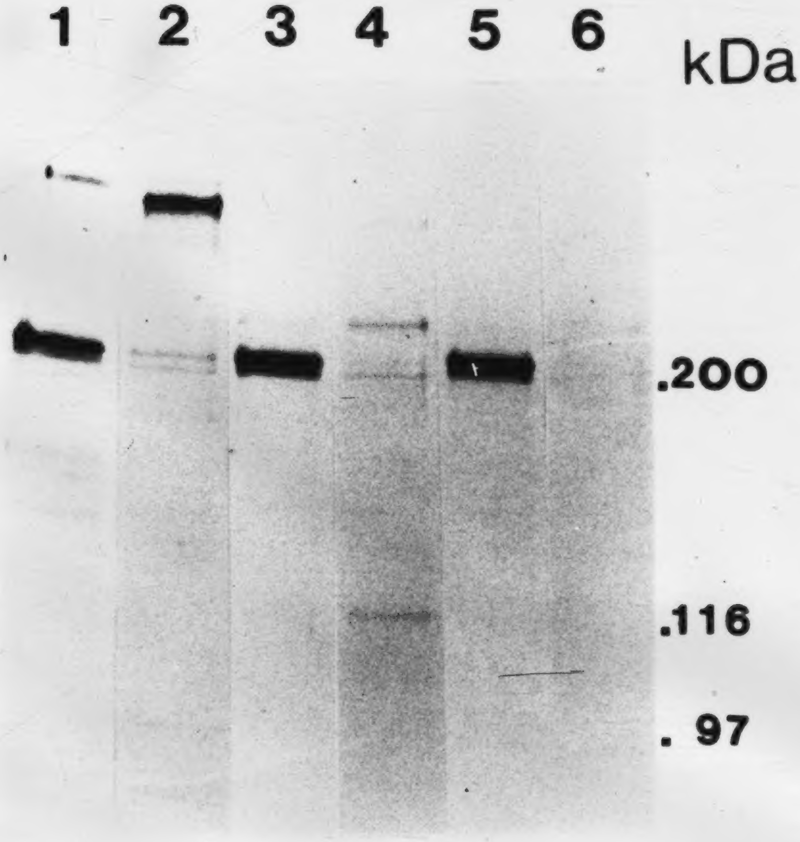
1
2
3
4
5
6
kDa
.200
.116
. 97

TABLE VI 1

SUMMARY OF ANTIBODIES USED IN THIS STUDY AND THEIR REACTIVITIES WITH PROTEINS OF THE LDL RECEPTOR FAMILY

All antibodies, except where indicated, were rabbit polyclonal IgG and are as described in the indicated references. "+" denotes reactivity between the antibody in the left-hand column and the antigen in the horizontal row, as determined by immunoblotting.

(1) as designated in the text

(2) IgG-2E1, kindly provided by Drs. R.C. Kowal, M.S. Brown, and J.L. Goldstein.

Antibodies	Proteins					References
	Chicken 95-kDa receptor	Chicken smaller LRP(1)	Chicken larger LRP(1)	Rat LRP	Bovine LDL receptor	
Anti-chicken 95-kDa oocyte receptor	+					3
Anti-chicken smaller LRP(1)		+				This study
Anti-chicken larger LRP(1)			+	+		This study
Anti-rat LRP	+		+	+		7
Anti-bovine LDL receptor	+	+			+	1
Anti-rat LRP(2) (murine monoclonal)	+		+	+		(2)

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Chapter VII

CHARACTERIZATION OF CHICKEN HEPATIC LDL RECEPTORS

INTRODUCTION

Following the studies described in Chapter VI, demonstrating i) interaction of vitellogenins from chicken and fish with both the LDL receptor and the LDL receptor-related protein from rat liver membranes and ii) the expression in chicken liver of a protein with properties identical to those of mammalian LRP (1), the present studies were performed in order to test the possibility that avian liver expresses a membrane protein with properties analogous to those of the LDL receptor in mammals. Previous work from our laboratory demonstrated that cultured chicken embryo fibroblasts express a 130-kDa cell surface protein that can bind apolipoprotein-B-containing lipoproteins and is involved in regulation of cellular cholesterol homeostasis (2,3), thus qualifying as a legitimate LDL receptor. In such studies, however, no emphasis was put on attempting to address the question of the possible expression and regulation of LDL receptors in chicken liver. We have now addressed such questions and have obtained additional information concerning the properties of the chicken LDL receptor.

MATERIALS AND METHODS

Materials, Animals, and Diets

All materials were from previously reported sources (4). White Leghorn laying hens and roosters were purchased from the Department of Animal Science, University of Alberta, and maintained as described (4). Hens of the "Restricted Ovulator" (R/O) strain were selected and maintained as described (5).

Isolation and Radioiodination of LDL

Rooster LDL (1.025 g/ml$<\rho<$1.063 g/ml) was prepared and radiolabeled with $Na^{125}I$ exactly as described in (6). Briefly, plasma collected by centrifuging blood at 3,000 x g for 10 min was subjected to ultracentrifugation for 36 hr at 200,000 x g at 4 °C. The floating lipoprotein fraction was collected, mixed with 150 mM NaCl, 0.2 mM EDTA (pH 7.4), 1 mM PMSF, and 2 µM leupeptin (buffer A; ρ=1.006 g/ml), and centrifuged again at 200,000 x g for 24 hr. At the end of this centrifugation step, the infranate was collected from the bottom half of the tube, adjusted to a density of 1.060 g/ml with solid KBr, and centrifuged at 200,000 x g for 20 hr at 4 °C. The top fraction resulting from this centrifugation step was recovered, dialyzed against buffer A and then subjected to equilibrium density gradient centrifugation. The KBr gradient was as follows, from bottom to top: 1.15 g/ml, 1.06 g/ml, and 1.02 g/ml solutions (3 ml each) of KBr in buffer A. Centrifugation was at 39,000 rpm at 4 °C for 12 hr in a Beckman SWTi 40 rotor. LDL was recovered at a position approximately 4 cm from the top of the tube. Recovered LDL was extensively dialyzed against buffer A and radiolabeled with ^{125}I by the monochloride method (6).

Preparation of Membrane Fractions and Solubilization of Membrane Proteins

All membrane fractions were obtained and membrane proteins solubilized in the presence of 1% Triton X100 as described (1). Where indicated, rat liver detergent extracts were subjected to DEAE-cellulose chromatography (7) and the fractions containing the peak of LDL receptor activity were collected and designated DEAE-cellulose fraction.

Electrophoresis and Blotting Procedures

All operations were as described in (1).

RESULTS AND DISCUSSION

In order to investigate the possible expression in chicken liver of the 130-kDa apo-B-specific receptor present on the surface of cultured chicken embryo fibroblasts (2,3), liver membranes were prepared, membrane proteins were solubilized, fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose in order to perfom ligand blotting experiments with ^{125}I-rooster LDL. As shown in Fig. VII 1, radiolabeled LDL bound to a protein from rooster liver membranes (lane 2) exhibiting electrophoretic mobility in the same M_r range as *bona fide* LDL receptors from bovine adrenal (lane 3) and rat liver (lane 4) membranes. The LDL-binding protein from chicken liver clearly showed an apparent molecular weight different from the chicken oocyte VTG/VLDL 95-kDa protein (lane 1), in agreement with previous studies with cultured chicken embryo fibroblasts (2,3). In those studies, evidence was presented that expression of the 130-kDa LDL receptor is controlled by intracellular levels of cholesterol or metabolic derivatives thereof, a situation first recognized and described in studies of the regulation of LDL receptor expression by human fibroblasts (8). Significant reduction of LDL receptor activity in cultured chicken embryo fibroblasts can be observed when cell monolayers are exposed to culture media containing either mixtures of cholesterol and oxygenated sterols (such as 25-OH-cholesterol) or relatively high amounts of apo-B-containing lipoproteins (2,3). Since this regulatory pathway is a hallmark of LDL receptors, the possibility that expression of the chicken hepatic 130-kDa protein identified in Fig. VII 1 could also be regulated by intracellular levels of lipoprotein-derived cholesterol was tested in the experiments described in Fig. VII 2. For these studies, we prepared liver membranes from either laying hens or hens of the "restricted ovulator" (R/O) strain. As shown previously (5), a single gene defect is responsible for dramatically reduced or absent binding capacity of the 95-kDa oocyte receptor for VTG and VLDL, respectively (4). As a result, these animals are incapable of completing vitellogenesis and thus fail to lay eggs. Because of the absence of functional growing oocytes, very high amounts of VLDL and VTG accumulate in the plasma of R/O hens, generating a hypercholesterolemic state that represents an ideal *in vivo* condition in which to investigate the responsiveness of hepatic LDL receptor expression to variations in plasma lipoprotein levels. Fig. VII 2 shows that, in ligand blotting experiments, ^{125}I-rooster LDL bound to the 130-kDa protein from membrane detergent extracts from the liver of laying hens (lane 3) and rats (lane 1) and, in addition, to the 95-kDa receptor from chicken oocytes (lane 2). In contrast, almost no detectable binding of labeled rooster LDL to the LDL receptor was observed when membrane proteins from R/O hen liver were loaded on the gel (lane 4) in amounts equal to samples from laying hen liver (lane 3). These results indicate that, in agreement with the situation described for cultured chicken embryo fibroblasts (2,3), hepatic LDL receptor activity is reduced in response to increased levels of cholesterol-carrying lipoproteins in the extracellular environment.

During studies with chicken embryo fibroblasts, an interesting difference between avian and mammalian LDL receptors was revealed (2), namely that, contrary to mammalian LDL receptors, the chicken 130-kDa protein does not recognize apolipoprotein-E, as demonstrated by its lack of interaction with β-VLDL. These observations acquire additional significance when examined together with the results described in Chapter VI of this thesis. Those studies have demonstrated that, while chicken VTG can be recognized by mammalian LDL receptors, it does not interact with the equivalent avian receptors, a situation identical to that observed for apo-E. It is reasonable to speculate that some of the structural features that allow other members of the LDL receptor family, such as the chicken oocyte 95-kDa receptor or LDL receptor-related proteins in mammals and chicken, to interact with VTG and apo-E may be absent in the 130-kDa chicken LDL receptor, thus resulting in concomitant loss of both VTG- and apo-E-binding activities. In order to investigate the possible existence of further differences between mammalian and avian LDL receptors, the ability of the latter to bind calcium ions was tested. As shown in Fig. VII 2, lanes 7 and 8, the chicken liver LDL receptor failed to bind any detectable amounts of $^{45}Ca^{2+}$. In contrast, strong binding of calcium ions was observed to the rat LDL receptor and LRP (lane 5), to the chicken oocyte VTG/VLDL receptor and smaller LRP (lane 6), and to the chicken liver LRP (lanes 7 and 8); additional unrelated calcium-binding proteins were also visualized after the long autoradiography shown in Fig. VII 2.

Studies on the role played by individual cysteine-rich repeats in the ligand binding domain of mammalian LDL receptors have demonstrated that repeat number 1 at the NH_2-terminus of the molecule is not directly involved in ligand interactions but is required for calcium binding (9). The possibility exists, yet to be demonstrated, that the lack of calcium binding exhibited by the chicken LDL receptor may be the result of the lack of or alterations in a cysteine-rich repeat equivalent to repeat 1 in the human LDL receptor. This may not only result in loss of calcium binding, but it may also cause the receptor to assume a conformation incompatible with VTG and/or apo-E recognition without affecting its ability to interact with apo-B. In agreement with this hypothesis, in experiments performed in our laboratory no interaction was demonstrated between the chicken LDL receptor and the monoclonal antibody IgG C7, which was shown to be directed against the first ligand binding repeat of the human LDL receptor (9). In contrast, the same antibody showed cross-reactivity with the chicken oocyte 95-kDa

VTG/VLDL receptor, which exhibits both calcium and apo-E-binding properties (Hayashi, K., and Schneider, W.J., unpublished observations). It is also possible, however, that differences at the level of the EGF precursor-like domain (which is also believed to be involved in calcium binding) between mammalian and avian LDL receptors may be responsible for the impaired Ca^{2+}-binding ability of the latter and the lack of VTG and apo-E recognition may result from an overal different organization of the modular binding domain of the receptor. While studies at the DNA/RNA level will be necessary to answer such questions, the present studies provide some initial working hypotheses and some useful experimental protocols to address these issues.

FIGURE VII 1

LIGAND BLOTTING OF HEPATIC LDL RECEPTORS

Chicken oocyte membrane Triton extract (lane 1; 50 µg of protein/lane), rooster liver membrane Triton extract (lane 2; 400 µg of protein/lane), bovine adrenal membrane Triton extract (lane 3; 350 µg of protein/lane), and estrogen-treated rat liver membrane DEAE-cellulose fraction (lane 4; 400 µg of protein/lane) were subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gradient gel, followed by transfer to nitrocellulose and ligand blotting in the presence of 4.4 µg/ml of ^{125}I-rooster LDL (198 cpm/ng). Autoradiography was for 40 hr at -70 °C. Symbols used indicate the following: ●, ▲-LRPs; ○-chicken oocyte VTG/VLDL receptor; △-LDL receptor

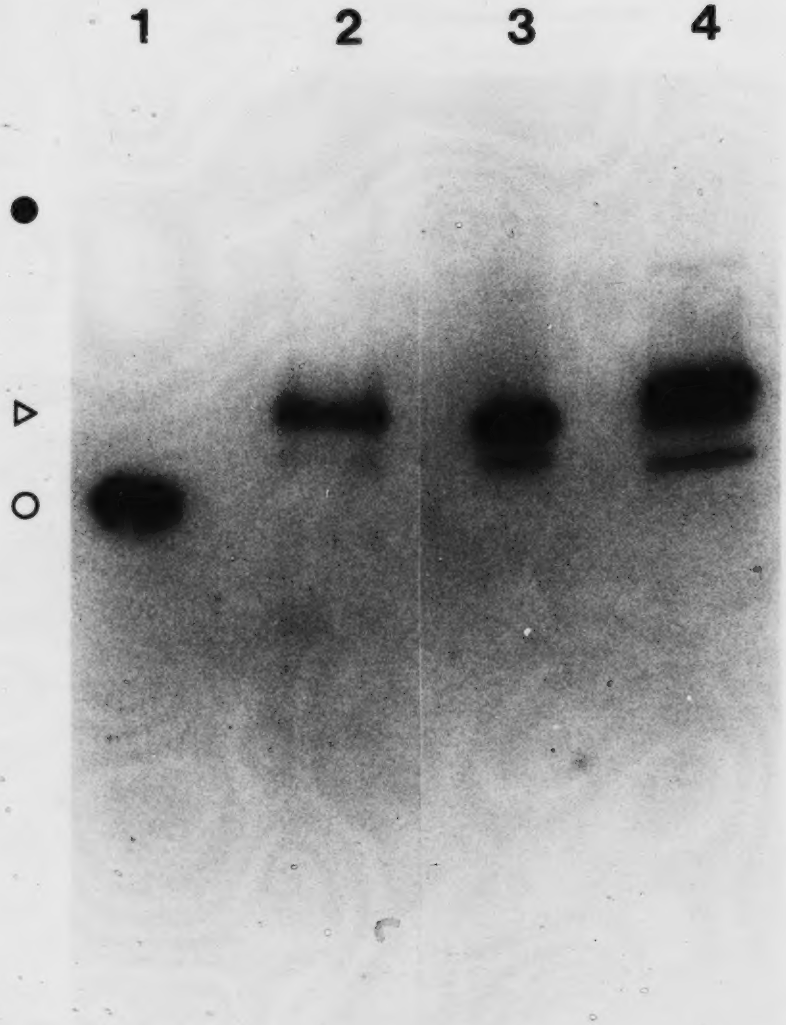
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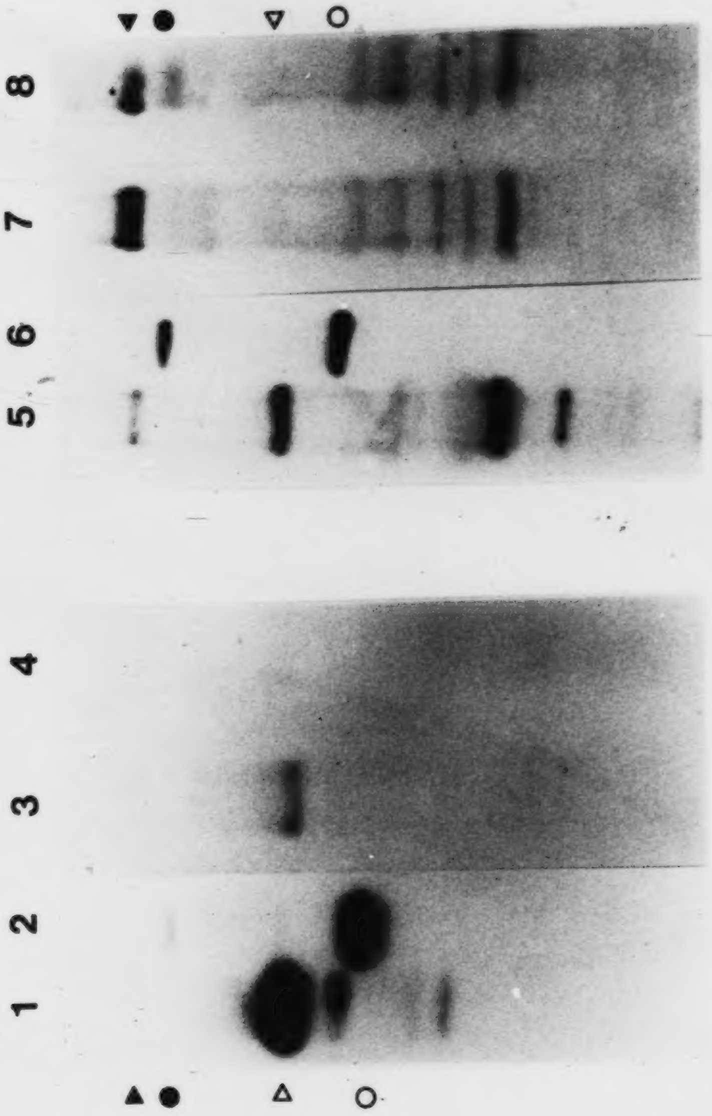
FIGURE VII 2

CHARACTERIZATION OF CHICKEN LIVER LDL RECEPTORS

Estrogen-treated rat liver DEAE-cellulose fraction (lanes 1 and 5; 300 µg of protein/lane), chicken oocyte membrane Triton extract (lanes 2 and 6; 40 µg of protein/lane), laying hen liver membrane Triton extract (lanes 3 and 7; 400 µg of protein/lane), and R/O hen liver membrane Triton extract (lanes 4 and 8; 400 µg of protein/lane) were subjected to electrophoresis on a 4.5 -18% SDS-polyacrylamide gradient gel, transferred to nitrocellulose and incubated either in the presence of 4.4 µg/ml of ^{125}I-rooster LDL (198 cpm/ng; lanes 1-4) or in the presence of $^{45}CaCl_2$ (lanes 5-8; 1 µCi/ml) as described in "Materials and Methods". Autoradiography was for either 16 hr (lanes 1-4) or 19 hr (lanes 5-8) at -70 ^{o}C. Symbols used are as described in Fig.VII 1.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▲ ● △ ○
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Chapter VIII

THE LDL RECEPTOR-RELATED PROTEIN AND THE α_2-MACROGLOBULIN RECEPTOR ARE IDENTICAL

INTRODUCTION

Recently, two large membrane proteins have been described which have the potential to function as receptors in endocytosis of two very diverse ligands. One of these proteins has been purified from human placenta (1,2), rat liver (3), and human fibroblasts (4) as the culmination of long-standing efforts to isolate and characterize the cell surface receptor for α_2-macroglobulin (α_2M). α_2M is a tetrameric plasma protein that forms inhibitory complexes with proteinases destined for rapid clearance from the circulation via hepatic receptors (5) The affinity-purified α_2M receptor (α_2MR) polypeptide has been assigned an M_r of 400,000 to 500,000 ; smaller components of 70 kDa to 90 kDa (1-4) are consistently co-purified.

The other large protein has emerged from studies aimed at elucidation of a receptor for intestinally derived lipoproteins termed chylomicron remnants. Cloning based on predicted homology with the low density lipoprotein (LDL) receptor resulted in the characterization of the so-called LDL receptor-related protein (LRP) (6). The predicted M_r of the unglycosylated human LRP polypeptide is 503,000 (6), and the intact glycoprotein has an apparent M_r of 500,000-600,000 as determined by electrophoresis on SDS-polyacrylamide gels (6-8). Single-chain LRP (LRP 600) is post-translationally proteolyzed into two non-covalently linked chains termed, according to their apparent sizes, LRP 515 and LRP 85 (8). LRP 515 constitutes most of the extracellular region of LRP 600, and is made up entirely of multiple copies of three types of repeats found in the LDL receptor (6,8). LRP 85 contains the cytoplasmic carboxyterminal portion of 100 residues harboring two sets of signals for endocytosis, the single membrane-spanning domain of LRP, and a small extracellular domain lacking repeat units of the type involved in ligand binding (6,8). When purified by standard procedures, most of the LRP exists in the two-chain form (8,9). Compatible with a role of LRP in lipoprotein clearance, lipoproteins enriched in apolipoprotein-E, the putative receptor-binding moiety of chylomicron remnants, have been shown by ligand blotting (10) and chemical crosslinking (11) to be recognized by LRP 515. However, as is the case for α_2MR, direct evidence for a defined role of LRP *in vivo* is lacking.

When we critically reviewed published work on α_2MR and LRP, we became intrigued by several strikingly similar observations about the proteins proposed to be the receptors for α_2M and chylomicron remnants, respectively. First, successful attempts to isolate α_2MR included detergent solubilization and ion-exchange chromatography (12) under conditions very similar to those used for partial purification of the LDL receptor (13) and of LRP (9). As well, a solid-phase binding assay developed for measuring solubilized LDL receptors (13) was used for α_2MR determinations (12,14). Second, in almost all purification schemes for α_2MR, a ~85-kDa component - reminiscent of LRP 85 - has been obtained, in particular when fibroblasts (4,12,14), placenta (1,2), and liver (3) were used as source for isolation; the 85-kDa chains are likely not involved in ligand binding (LRP, ref. 9; α_2MR, ref. 3). Third, we were struck by the fact that normal rat kidney cells served as a prominent experimental system for investigations of both LRP (9) and α_2MR (1,12), apparently because of high levels of expression of these proteins. Fourth, the large and similarly sized α_2MR and LRP chains are known to be cysteine-rich, as concluded either from direct sequence analysis (LRP, ref. 6) or from amino acid analysis (α_2MR, ref. 15) and decreased electrophoretic mobility on SDS-polyacrylamide gels upon reduction by sulfhydryl compounds (α_2MR, ref. 3). Fifth, both receptors bind Ca^{2+} with high affinity, and binding of the proposed physiological ligands is Ca^{2+}-dependent (3, 10, 15). Interestingly, binding of Ca^{2+} to both α_2MR (15) and LRP (6,7) has been demonstrated by incubation with $^{45}Ca^{2+}$ following transfer of the proteins from SDS polyacrylamide gels to nitrocellulose, and is restricted to the large chains (6,7,15). Sixth, the *in vivo* clearance of α_2M as well as of chylomicron remnants is known to occur primarily through hepatic receptor activity (5,16), with a $t_{1/2}$ of less than 5 min (5,17), concomitant with the liver being the source of choice for purification of the proteins (3,9). We now have directly tested whether or not the two polypeptides are identical. We have compared the reported chemically determined amino acid composition of α_2MR with that of LRP as derived from its known amino acid sequence: they are, within margin of error for the analysis of α_2MR, identical. In addition, we have obtained direct evidence of the ability of α_2M to interact with LRP by showing that α_2M/LRP complexes can be immunoprecipitated using antibodies directed against α_2M. Finally, we present evidence that human α_2M can be recognized by the chicken oocyte 95-kDa VTG/VLDL receptor, a protein that has been shown to be related to human LRP (Chapter VI).

MATERIALS AND METHODS

Materials, Animals, and Diets

Human α_2-macroglobulin (cat. no. M-7151) and goat anti-human α_2-macroglobulin IgG (cat. no. M-0140) were from Sigma; rabbit anti-goat IgG (cat. no. 06060082) and goat anti-rabbit IgG (cat. no. 06120081) were from Cappel; all other materials were from previously reported sources (Chapters II-VI). Rats and white Leghorn laying hens were maintained as described in Chapter VI.

Immunoprecipitation of α_2-macroglobulin/LRP complexes

Preformed immune complexes were prepared by incubating 150 μg of goat anti-human α_2-macroglobulin IgG (in PBS; 50 μl) and 300 μg of rabbit anti-goat IgG (in PBS; 70 μl) for 4 hr at room temperature with gentle mixing at regular intervals. Control preformed complexes were prepared in the same way using 150 μg of goat anti-rabbit IgG. A 50-μl aliquot of the preformed complexes was mixed with 150 μl of a mixture containing 500 μg of protein of solubilized rat liver membranes and 45 μg of human α_2-macroglobulin that had been activated for receptor binding by treatment with methylamine as reported (2); this mixture had been previously incubated for 4 hr at 4 °C. Incubation was prolonged for 2 hr at 4 °C, after which immunoprecipitates were collected by centrifugation for 5 min in an Eppendorf microfuge. Pellets were separated from supernatants and washed 4 times with PBS containing 0.05% (v/v) Triton X-100, then dissolved in 80 μl of a 1:1 mixture of 2 x O'Farrell's buffer and 6 M urea. Samples were then subjected to SDS-polyacrylamide gel electrophoresis on a 4.5-18% gradient gel, followed by transfer to nitrocellulose.

Other Methods

SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose, immunoblotting, and ligand blotting were performed as described previously (Chapters II-VI). The monoclonal antibody 2E1 against rat LRP was a gift of Drs. R.C. Kowal, J.L. Goldstein, and M.S. Brown and was used in immunoblotting experiments as described in Chapter VI. Chicken VTG (Chapter II) and VLDL (Chapter III) were prepared as described previously.

RESULTS AND DISCUSSION

Motivated by the aforementioned observations of very similar properties of LRP and α_2MR, and triggered by the recent report of the amino acid composition of human α_2MR (15), we have investigated whether α_2MR and LRP might in fact be the same protein. Inasmuch as the amino acid composition of human α_2MR was determined from a fraction that contained only the large polypeptide (estimated M_r 440,000) following separation from smaller polypeptides (such as the 85-kDa fragment) (15), we compared its composition with that of the large chain of human LRP, LRP 515. As discussed in ref. 15, the method used for determination of the number of residues/molecule α_2MR chain was subject to considerable error; we have therefore compared the numbers of mol% for each amino acid residue as reported for α_2MR (15) and calculated for LRP 515 (6,8), respectively. As Fig. VIII 1 clearly demonstrates, the amino acid compositions of the two polypeptides are remarkably similar, if not identical; deviations of α_2MR from LRP are well within the margins of error for chemical analysis of such a large molecule, and thus we tentatively conclude that the polypeptide proposed to be α_2MR is the same as that termed LRP.

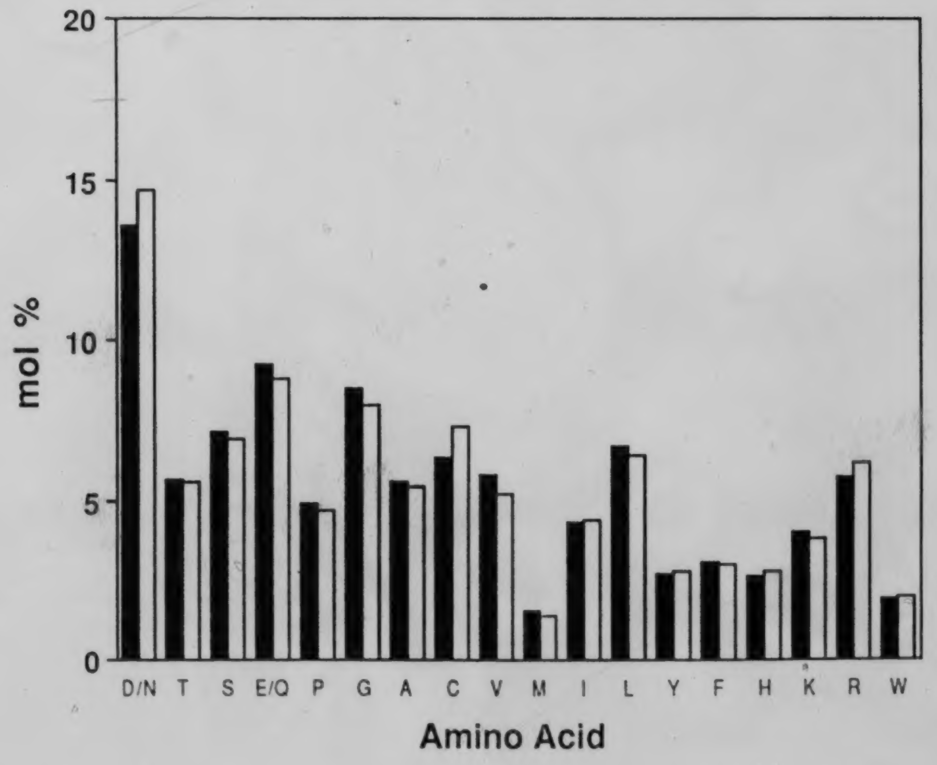
In order to obtain direct proof of a molecular interaction between α_2M and LRP, we incubated aliquots of rat liver membrane Triton extract in the presence of human α_2M that had been activated for receptor binding by previous incubation with methylamine (α_2M:m) and then subjected the incubation mixtures to immunoprecipitation reactions with either antibodies against α_2M or nonspecific antibodies (treatment with methylamine causes cleavage of an internal thiol ester bond in each subunit and a conformational change leading to the exposure of a previously concealed receptor recognition site). The products of the immunoprecipitation reactions were then subjected to SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose, and immunoblotting with a monoclonal antibody directed against rat LRP (Chapter VI) Fig. VIII 2 shows that the immunoprecipitate obtained after incubation of rat liver membrane extract first with human α_2M and then with anti-human α_2M IgG contained a protein that was recognized by the anti-rat LRP IgG (lane 2) and which exhibited an electrophoretic mobility compatible with LRP (lane 1). Such protein was not visualized in immunoprecipitates obtained from control incubations in which either a nonspecific antibody was used (lane 3) or α_2M was omitted (lane 4). Other bands visible in all lanes are due to interaction of ^{125}I-protein A with IgG heavy and light chain complexes present in all immunoprecipitates. These results show that antibodies against human α_2M can coimmunoprecipitate LRP in addition to α_2M, thus demonstrating that the two proteins can physically associate with each other. Together with the comparison of the amino acid composition of LRP and α_2MR shown in Fig. VIII 1, these findings provide direct evidence in support of our conclusion that these two recepors are indeed the same molecular entity.

As shown in Chapter VI, we have recently observed that chickens express two distinct forms of LDL receptor-related proteins, both of which bind vitellogenin, a ligand which is also recognized by mammalian LRPs. In view of the present demonstration of the identity of LRP with the α_2M receptor, we next tested if human α_2M could bind to chicken LRPs and/or the functionally related chicken oocyte 95-kDa VTG/VLDL receptor. Fig. VIII 3 shows that in ligand blotting experiments with chicken ovarian membrane extracts, ^{125}I-α_2M bound to the 95-kDa receptor (lane 1), but to neither of the two LRPs shown to be present in these membrane fractions (Chapter VI). Binding of ^{125}I-human α_2M to the VTG/VLDL oocyte receptor was specific, in that it could be reduced by performing our incubations in the presence of a large excess of either unlabeled VLDL (lane 2) or unlabeled VTG (lane 3), or in the presence of EDTA (lane 4). A calcium-requirement for binding to its receptor is a known property of α_2M (15). These findings are in agreement with immunological and biochemical studies that showed that there is extensive cross-reactivity between mammalian LRPs and chicken follicular VTG receptors (Chapter VI); however, these studies failed to demonstrate interaction between human α_2M and chicken LRPs. Two possible explanations can be put forward: first, chicken LRPs may not be able to recognize α_2M under these experimental conditions, or second, chicken LRPs may not recognize human α_2M but may still be able to interact with chicken α_2M. In order to address such important issues, it will be necessary to develop conditions for the purification of chicken α_2M that will allow a direct comparison of the properties of the human and avian proteins. These studies are expected to provide important answers to the question of how such apparently diverse ligands can interact with common receptors. In particular, does the identity of LRP and α_2MR extend to the mode of ligand recognition; *i.e.*, do the ligands recognized by these receptors have common structural features, as suggested by destruction of receptor-recognition upon derivatization of lysine residues in VTG (18) apo-E (19) and α_2M (27)? If so, are there other plasma proteins with recognition sites for α_2MR/LRP? With appropriate specific probes for detailed analysis of the α_2MR/LRP locus and structure/function relationships of the protein forthcoming, future investigations are hoped to answer these and other questions.

FIGURE VIII 1

COMPARISON OF THE AMINO ACID COMPOSITION OF HUMAN α_2MR WITH THAT OF HUMAN LRP

The human α_2-macroblobulin receptor amino acid composition (15) (open bars) was compared with the amino acid composition of human LRP (residues 1-3924) as deduced from its primary sequence (6,9) (filled bars). The mole % for each of the indicated amino acids are presented. D/N denotes the sum of Asp + Asn, and E/Q that of Glu and Gln, respectively. The order from left to right on the abscissa is the same as that in Table I in ref. 15.


20
15
10
5
0
mol %
D/N
T
S
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I
L
Y
F
H
K
R
W
Amino Acid

FIGURE VIII 2

COIMMUNOPRECIPITATION OF α_2M:m AND LRP

Rat liver membrane Triton extract (500 µg of protein), prepared as described in "Materials and Methods" was incubated for 4 hr at 4 °C with (lanes 2 and 3) or without (lane 4) 45 µg of human α_2M that had been activated by treatment with methylamine (2), in a final volume of 150 µl. Then, each incubation mixture received a 50-µl aliquot of either preformed immune (lanes 2 and 4) or nonimmune (lane 3) complexes prepared as described in "Materials and Methods". Tubes were further incubated for 2 hr at 4 °C and immunoprecipitates were collected by centrifugation. After four washes with a phosphate-buffered saline solution containing 0.05% Triton X-100, immunoprecipitates were dissolved in 80 µl of a solution containing 3 M urea, 62.5 mM Tris-HCl (pH 6.8), 1% SDS, and 10% glycerol. SDS-polyacrylamide gradient gel electrophoresis of dissolved immunoprecipitates and the following transfer to nitrocellulose and immunoblotting with monoclonal antibody 2E1 were as described in "Materials and Methods". Lane 1 shows the result of immunoblotting of control rat liver membrane extract (200 µg of protein) with monoclonal antibody 2E1. Bands (in lanes 2-4) below the LRP band are from interaction of ^{125}I-protein A with IgG molecules present in the immunoprecipitates.

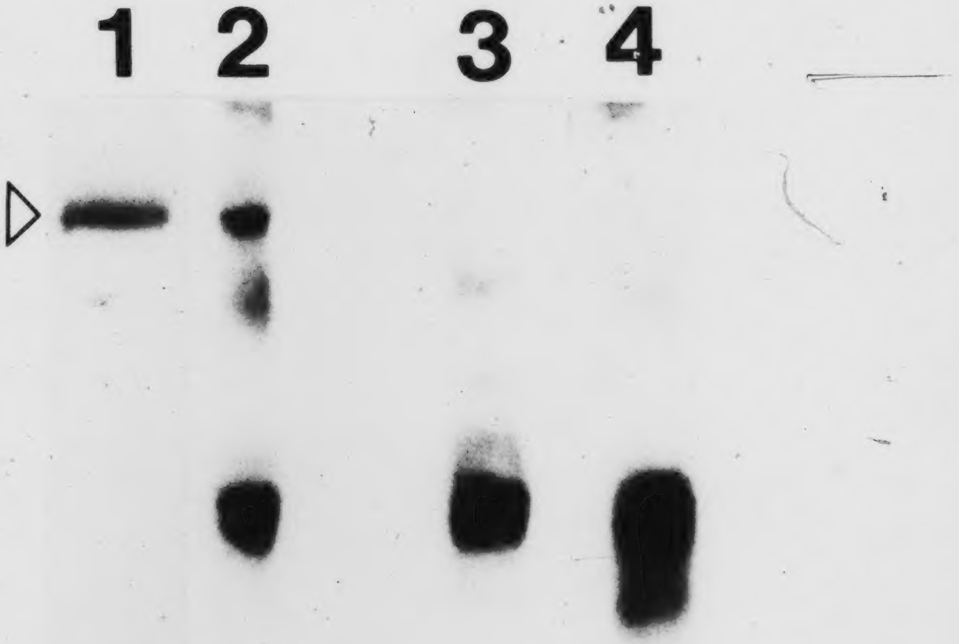
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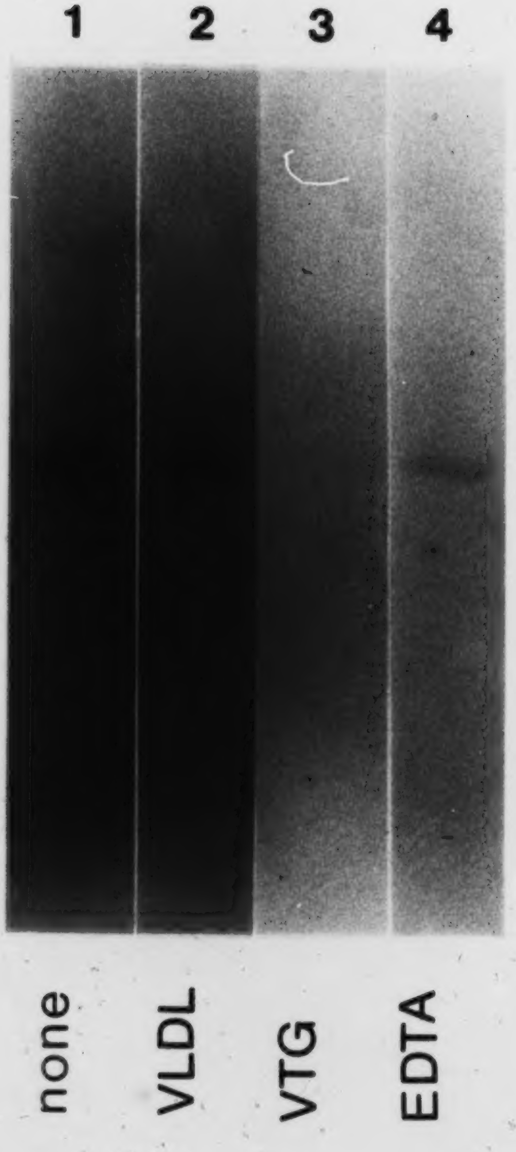
FIGURE VIII 3

BINDING OF ^{125}I-HUMAN α_2M:m TO CHICKEN OVARIAN MEMBRANE PROTEINS

Chicken ovarian membrane Triton extract (250 µg of protein/lane) was subjected to SDS-polyacrylamide gel electrophoresis on a 4.5-18% gradient gel, followed by transfer to nitrocellulose. All strips were incubated with 2.0 µg/ml of ^{125}I-human α_2M:m in the presence of the following additions: lane 1, none; lane 2, 300 µg/ml of chicken VLDL; lane 3, 100 µg/ml of chicken VTG; and lane 4, 10 mM EDTA.


1
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3
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none
VLDL
VTG
EDTA

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Chapter IX

DISCUSSION

This thesis was initiated with the goal of obtaining detailed biochemical information about the plasma membrane receptors that promote internalization of lipoproteins, particularly vitellogenin, into the growing oocytes of oviparous animals. Beside providing insight into some of the mechanisms regulating the growth of these giant cells, such as receptor-mediated endocytosis, these studies were also expected to put forward new concepts relative to the features of lipoprotein receptors in nonmammalian vertebrates, including their possible relatedness to mammalian low density lipoprotein receptors. In this context, several novel findings obtained during these studies on the regulation of vitellogenesis in birds and during investigations in other laboratories on novel lipoprotein receptors in mammals provided important information that can be expected to facilitate the understanding of the physiological processes that are mediated by these receptors and the molecular basis of their biochemical interactions with specific ligands. Moreover, new hypotheses were formulated, suggesting a role for oocyte lipoprotein receptors as predecessors of evolutionarily younger molecules characterized by new functional properties.

One group of results provided the first detailed characterization of three different members of the VTG-receptor family from avian, amphibian, and fish oocytes (Chapters II-V). The characterization of three different members of this group of receptors was instrumental in that it allowed to obtain and verify the notion that vitellogenesis is tightly regulated at the level of receptor-mediated internalization of yolk precursors. This is achieved through the action of a system of ligands and receptors that have been faithfully carried over from species to species under strong evolutionary pressure to remain virtually unchanged. All three characterized receptors share key structural features that allow them to be functionally equivalent with respect to ligand recognition. Such a situation allowed the demonstration that the determinants recognized by each one of the VTG receptors within the VTG molecules are localized to the NH_2-terminal portion of the ligands, a region referred to as lipovitellin I (Chapters IV-V). During the course of those studies, as knowledge of the properties of VTG receptors became increasingly more detailed, several common properties among these receptors and mammalian LDL receptors were revealed, including immunological cross-reactivity and, in the specific case of the chicken VTG receptor, ability to interact with apolipoprotein-B-containing lipoproteins such as VLDL and LDL (Chapter III). In agrement with these observations, direct demonstration of binding of vitellogenins from chicken and fish to the rat and bovine LDL receptor was obtained (Chapter VI), indicating that in viviparous organisms the loss of vitellogenin polypeptides was not accompanied by the complete loss of the VTG receptor; rather, a novel version of this gene product seems to have evolved into the LDL receptor and to have retained one of the properties of avian VTG receptors, namely the specificity for apo-B. Given the known ability of mammalian LDL receptors to recognize, in addition to apo-B, a second important plasma apolipoprotein, apo-E, and based on the observation that the chicken oocyte VTG/VLDL receptor can also interact with apo-E even though this apolipoprotein is not synthesized in birds (1), we suggested that VTG may represent the chicken counterpart to mammalian apo-E. This hypothesis was supported by the demonstration that VTG can effectively inhibit the binding of human apo-E to the chicken 95-kDa oocyte receptor (1). These results acquire additional significance when considered together with the second, complementary, group of findings reported in this thesis, namely the characterization of a pair of lipoprotein receptors in birds with properties similar, if not identical, to the recently identified mammalian LDL receptor-related protein.

LRP was initially identified during the course of studies aimed at investigating further the observation that levels of apo-E-containing lipoproteins, such as chylomicron remnants, are not elevated in individuals or laboratory animals with defective LDL receptors (2), a situation suggestive of a route different from the apo-B/E-specific LDL receptor for removal of these particles from the circulation (3). These studies resulted in the cloning of a gene encoding a product with properties closely resembling those of the LDL receptor (3-5). Initial results from investigations into the possible function(s) of this gene product have indeed started to support LRP's proposed role as an apo-E-specific receptor (4-6), but in the same time have failed to provide any *in vivo* demonstration that this cell surface protein is involved in uptake of chylomicron remnants by the liver.

The results presented in this thesis have shed new light into the understanding of the role(s) played by this fascinating protein and have identified a new powerful system for the study of its properties. The laying hen expresses not one but two forms of low density lipoprotein receptor-related protein, both of which were identified by their ability to interact with vitellogenin. Interestingly, VTG is also recognized by mammalian LRP (Chapter VI). These results have potentially important implications because i) chickens neither synthesize apo-E (7) nor deliver intestinally-derived lipoproteins through the lymphatic system like mammals do with chylomicron remnants (8), and ii) mammals do not synthesize VTG. Thus, together with the results obtained from studies of the 95-kDa oocyte VTG/VLDL receptor, these observations strengthen our suggestion that VTG may represent in avian species a counterpart to mammalian apo-E. Given the role of VTG as precursor of yolk components and the high level of expression of LRP in the growing follicles, it is possible that LRP might be the product of an ancestral gene designed to ensure an important event during oogenesis, i.e. yolk deposition. A modern, possibly acquired, function of hepatic LRP could be its involvement in plasma clearance of non-yolk lipoproteins.

Although the identification of the role of LRP during vitellogenesis in oviparous animals is an important finding toward obtaining knowledge of how this important process is regulated, it is the demonstration of the evolutionary relatedness between VTG receptors and mammalian lipoprotein receptors that is of particular significance. The results of this thesis clearly demonstrate that, in this particular context, the answer to the general question of what came first, the ligand or the receptor, is that the receptors for VTG (probably the first type of lipoprotein to appear) were available well before modern lipoproteins such as LDL or VLDL became available and these new ligands evolved with conserved features that allowed them to take advantage of the presence of existing receptors, even when apparently unrelated processes were taking place. This concept is perhaps most dramatically supported by the observation of the identity between LRP and the independently characterized α_2-macroglobulin receptor (Chapter VIII and ref. 9). No obvious similarity has been shown between α_2-macroglobulin and VTG or other apo-B- and apo-E-containing lipoproteins, yet they bind to the same molecule. This pluripotency for ligand recognition may suggest that, both in the liver and in the oocyte (two sites characterized by a similar predisposition to active internalization of a broad variety of molecules) LRP may serve as a multifunctional receptor for ligands sharing common traits that have yet to be satisfactorily characterized.

In conclusion, the present studies have not only opened up new vistas regarding the molecular bases of the regulation of vitellogenesis, but have at the same time provided new information and new hypotheses pertinent to the understanding of the function of the increasingly expanding family of LDL receptor-related genes.

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